Hepatitis B virus specific adoptive immune transfer in living liver donation and characterization of a prophylactic/therapeutic vaccine against Hepadnaviral infection

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Abbreviations

aa	amino acid
Anti-HBc	Antibodies to Hepatitis B virus core antigen
Anti-HBs	Antibodies to Hepatitis B virus surface antigen
DNA	Deoxyribonucleic acid
GST	Glutathione S-transferase
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B envelope antigen
HBIg	Hepatitis B specific immunoglobulin
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
IFN-γ	Interferon-gamma
IU	International units
kb	kilo base pairs
l	Liter
L	HBV large surface protein
LLD	Living liver donor
M	HBV middle surface protein
MHC	Major histocompatibility complex
MIR	Major immunodominant region
PAGE	Polyacrylamide gel electrophoresis
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
S	HBV small surface protein
SI	Stimulation index
VLPs	Virus like particles
WHcAg	Woodchuck hepatitis core antigen
WHV	Woodchuck hepatitis virus

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1. Introduction

1.1. Hepadnaviridae

Hepatitis B virus (HBV) is a member of the family Hepadnaviridae and belongs to the Orthohepadnavirus genus. The genome of HBV is circular and partially double-stranded with a size of only about 3.2 kb. The family of Hepadnaviridae including the mammalian (Orthohepadnavirus) and avian (Avihepadnavirus) species have a common structure and lead to persistent virus infection. Table 1 sums up members of Hepadnaviridae (Blumberg et al., 1967; Summers et al., 1978; Marion et al., 1980; Mason et al., 1980; Sprengel et al., 1988; Testut et al., 1996; Lanford et al., 1998; Chang et al., 1999; Warren et al., 1999) and their natural hosts (Tab. 1).

Table 1: Hepadnaviridae and their natural hosts

Virus	Host	Literature
Genus *Orthohepadnavirus*		
Hepatitis B virus (HBV)	*Homo sapiens*	Blumberg et al., 1967
Woodchuck hepatitis virus (WHV)	*Marmota monax*	Summers et al., 1978
California ground squirrel hepatitis virus (GSHV)	*Spermophilus beecheyi*	Marion et al., 1980
Arctic ground squirrel hepatitis virus (AGSHV)	*Spermophilus parryii*	Testus et al., 1996
Orang-Utan hepatitis virus (OHV)	*Pongo pygmaeus*	Warren et al., 1999
Woolly monkey hepatitis B virus (WMHBV)	*Lagothrix labotricha*	Lanford et al., 1998
Genus *Avihepadnavirus*		
Duck hepatitis B virus (DHBV)	*Anas domesticus*	Mason et al., 1980
Heron hepatitis B virus (HHBV)	*Ardea cineria*	Sprengel et al., 1988
Snow goose hepatitis B virus (SGHBV)	*Anser caerulescens*	Chang et al., 1999

1.2. The viral morphology

Hepatitis B virus virions, also called Dane particles (Blumberg et al., 1967; Dane et al., 1970; Blumberg, 1977), have a diameter of approx. 42 nm (Fig. 1). The envelope of the virus consists of three proteins in a glycosylated and non-glycosylated form: The large (L), the middle (M) and the small (S) protein. The S protein (226 amino acids) is encoded by s gene. PreS2 and s genes encode the M protein (281 amino acids), while preS1, preS2 and s encode L protein (389 or 400 amino acids, depending on the subtype). The HBV genome is encapsulated by the nucleocapsid (core) protein (HBcAg) and bears viral DNA. The HBV core protein consists of 183 or 185 amino acids depending on the genotype and is highly conserved among all HBV genotypes (Chain et al., 2005). The fundamental building unit of HBV is formed by dimerization of two core proteins, oligomerization of the dimers and formation of two types of capsids: Capsids consisting of 90 dimers associated in a icosahedral symmetry (T = 3) of about 30 nm in diameter and capsids consisting of 120 dimers associated in an icosahedral symmetry (T = 4) of about 34 nm in diameter (Zheng et al., 1992; Wynne et al., 1999). Virus particles are present in very large quantities in peripheral blood of infected patients (up to 10^{11} particles/ml). Further, empty spherical or filamentous particles, approx. 22 nm in diameters, are present (Fig. 1). The spherical particles are organized as an octahedral sphere containing 48 S-dimer subunits and the filamentous particles consist of the same diameter but differ in the length (Gilbert et al., 2005).

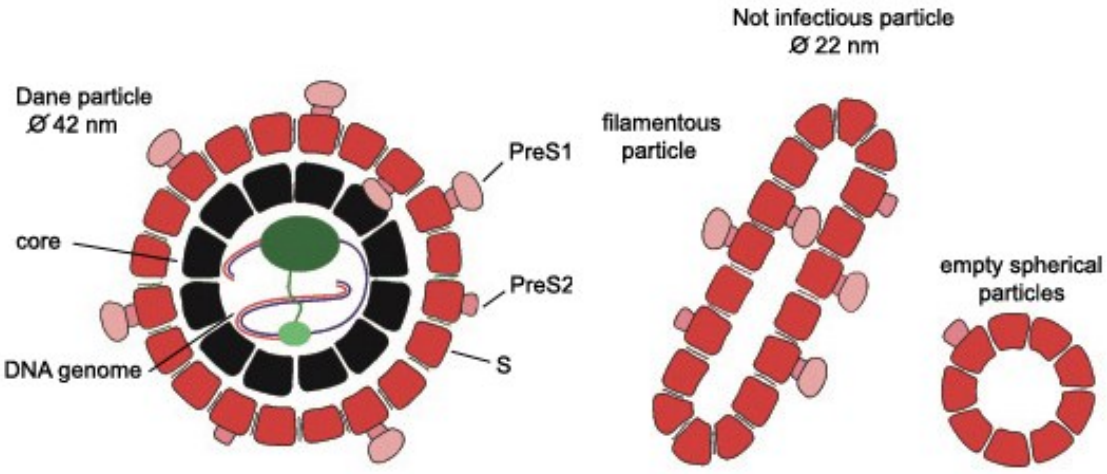

Figure 1: The composition of infectious and non-infectious particles of Hepatitis B virus. On the left: infectious Dane particle with PreS1, PreS2 and S proteins. The HBV genome is encapsulated by the core protein. On the right: Non-infectious filamentous and spherical particles. Figure according to Gerlich (Gerlich et al., 2009).

1.3. The viral genome

The HBV genome is approx. 3.2 kb long and is one of the smallest replication-competent virus genomes. HBV is classified into eight genotypes A-J, each with a distinct geographic distribution (Lin et al., 2011). The partially double stranded DNA genome of HBV contains a full length negative sense strand that is complementary to pregenomic viral RNAs. The viral genome encodes four overlapping open reading frames for the transcription of surface, core, polymerase, and X proteins. The viral polymerase (P) protein is covalently attached to the 5` end of the minus strand (Gerlich et al., 1980). The virion DNA is linear but arranged as a circle with a specific gap or nick in the negative-sense strand (Gerlich et al., 2009).

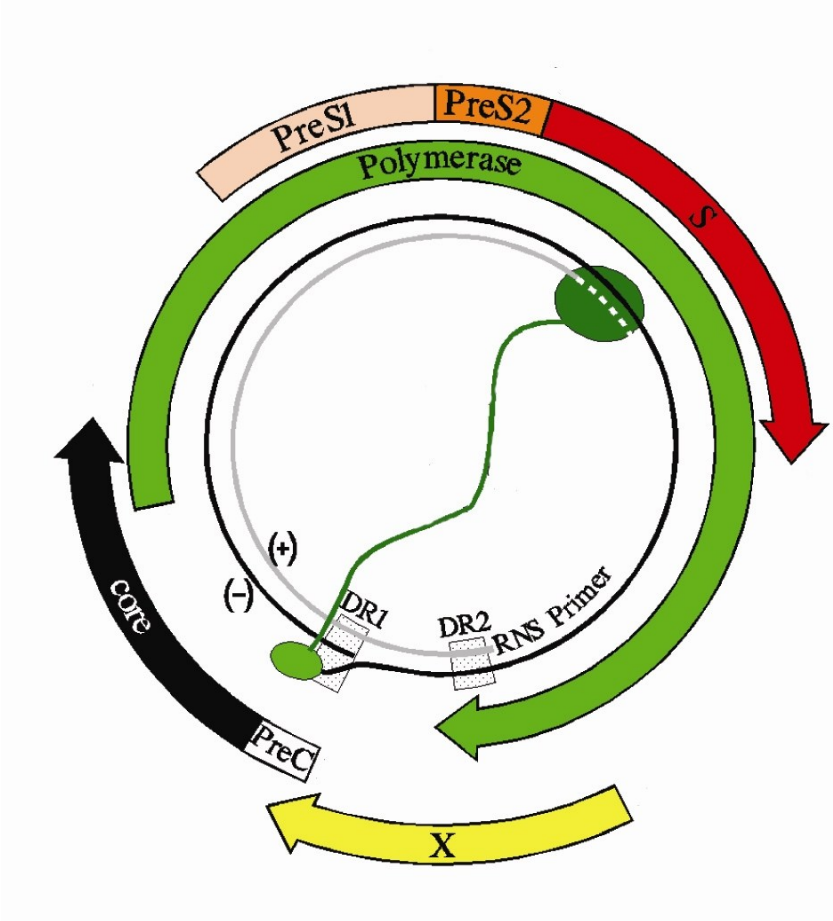

Figure 2: Genome scheme of HBV.
The four encoded translational reading frames of the DNA sequence are C-core, S-surface proteins, P-polymerase, and X protein. The core and polymerase open reading frames are in the same orientation, but the S translational reading frame overlaps these reading frames. Figure according to Gerlich (Gerlich et al., 2009).

1.4. The viral replication cycle

The Hepadnaviral replication takes place in hepatocytes. Other types of cells have been found to allow HBV replication to a lesser degree (Parvaz et al., 1987; Pontisso et al., 2008). Virus entry of Hepadnaviridae into hepatocytes is dependent on N terminus of the large (L) surface antigen (Glebe, 2006; Leistner et al., 2008; Schulze et al., 2010; Zhang et al., 2011a). Recently, HBV attachment and entry into hepatocytes has been visualized (Meier et al., 2013; Schieck et al., 2013). Simultaneously another group of researchers identified the sodium taurocholate cotransporting polypeptide (NTCP) as a hepatocyte receptor, which allows the entry of the virus (Yan et al., 2012; Zhong et al., 2013). After viral entry the capsid moves to the cell's nucleus where the viral nucleic acid is released and a covalently closed circular (ccc) DNA formed. This plasmid-like DNA serves as template for transcription of viral RNAs (Bock et al., 1994). The transcripts from the cccDNA consist of: Two species of 3.5 kb long RNAs (pregenomic and precore) and the 2.4 kb, 2.1 kb and 0.7 kb long subgenomic RNAs (Liang, 2009). After transport to the cytoplasm the viral RNAs are translated by ribosomes to core, the surface proteins (S, M and L) and the X-protein. The surface proteins differ in their N-terminal sequences that are longer in case of L and M protein. They consist of a common S domain, M consist of additional preS2 and L additional preS2 and preS1 domains. These proteins assemble the viral envelope. Further, surface proteins can be secreted as non-infectious filamentous and/or spherical particles that do not contain a functional nucleocapsid. The pregenomic (pg) RNA is both a template for core and for viral DNA-polymerase. The viral DNA-polymerase binds the pg RNA and induces the packaging of pg RNA into the already translated core protein. The core protein forms the basis of the capsid and also plays an active role in binding and packaging of pg RNA (Bock et al., 2001). After transcription of pg RNA to the minus strand DNA and subsequent positive strand synthesis, the capsid interacts with L-proteins and finally acquires the envelopment of core with the surface proteins. Finally, viral particles are secreted into the extra-cellular milieu via multivesicular bodies.

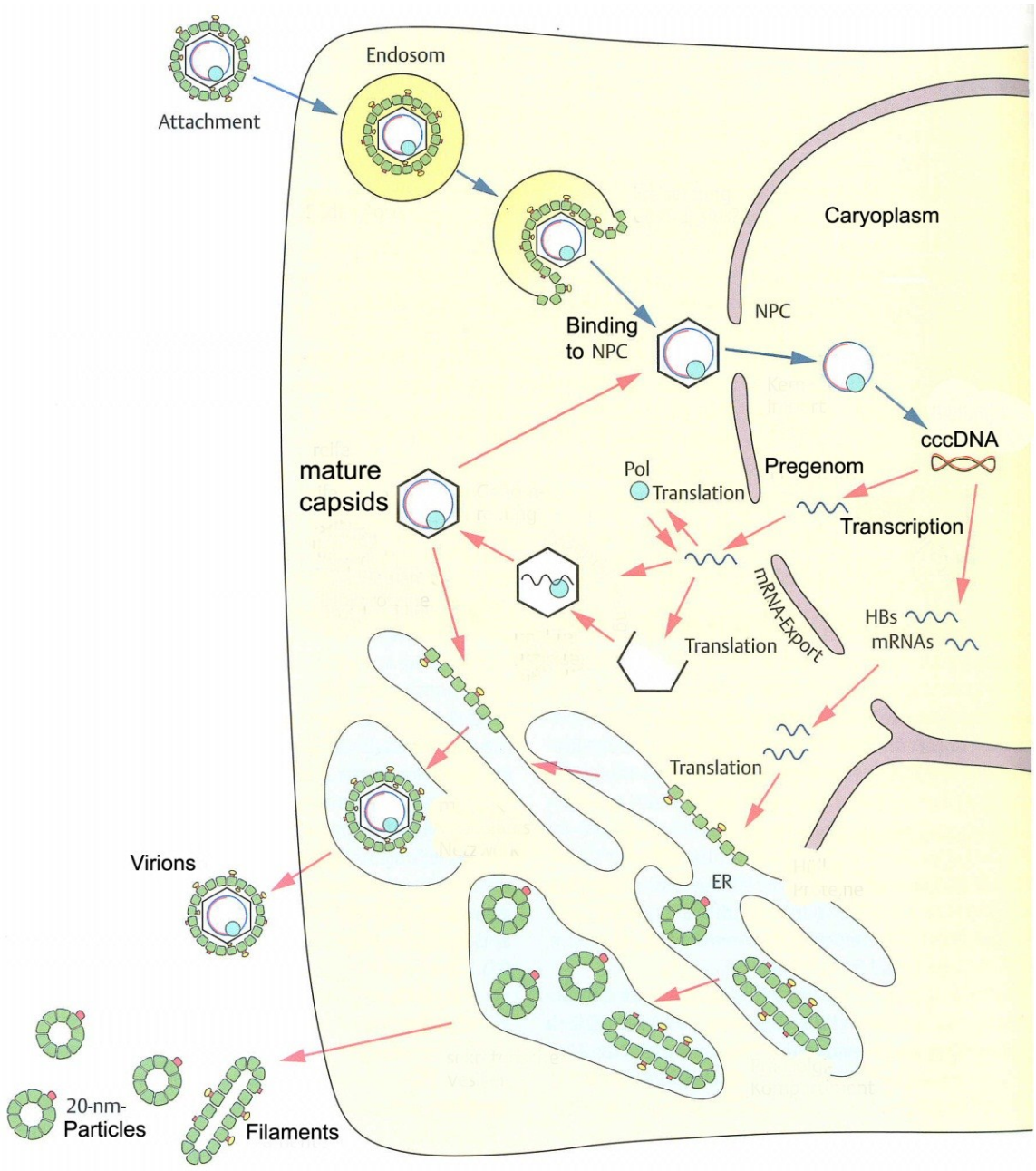

Figure 3: Live cycle of HBV.
Figure according to Gerlich (Gerlich et al., 2009).

1.5. Clinical course and immune pathogenesis of HBV infection

Hepatitis B virus is transmitted by contact with contaminated blood or other body fluids (i.e. semen and vaginal fluid). The incubation time can vary from about 30 to 180 days. HBV persists for widely variable periods of time. 65% of infections in young persons and adults are asymptomatic. In 35% the infection results in liver inflammation. The symptoms of the acute hepatitis are icterus, enlarged liver and occasionally disruption of blood development. The symptomatic acute infection persists normally two to three weeks. After this period the infection cures or converts into a chronic infection in 5 to 10% of all infected persons (WHO, 2002). However, infection in new-borns drifts in 90% to a chronic disease. In young children chronic infection occurs in 50% of infections. 60% of the chronically infected persons remain asymptomatic with a chronic persistent hepatitis (CPH). The remaining chronic infections result in chronic active hepatitis (CAH). CAH can spontaneously be transformed to CPH (Modrow et al., 2003).

After entrance into the body the virus reaches the liver and infects hepatocytes. Activation of the innate immune response to HBV is weak, however, viral clearance and liver pathology are largely mediated by the adaptive immune response (Chisari et al., 2010) Prior to virus replication in hepatocytes most probably Kupffer cells recognize HBV patterns and activate NF-κB and proinflammatory cytokines (Hosel et al., 2009). Further, interleukin-6 seems to be responsible for suppression of HBV early after infection of the hepatocytes while it activates mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK), which down regulate expression of hepatocyte nuclear factor 4α (HNF4α) and HNF1α, the key transcription factors regulating HBV gene expression (Quasdorff et al., 2008). However, the lack of interferon response in the early phase of infection is responsible for the typical widespread expansion of HBV in the liver. In vivo experiments in transgenic mice showed, that interferon-α and -β reduce expression of HBV genes in the early infection phase (Wieland et al., 2000).

Disease pathogenesis and viral clearance are largely mediated by the adaptive immune response. Adaptive immune response, particularly the HBV-specific CD8 T cell response, is strong and multispecific in patients with self-limited infection, but usually weakly detectable in patients who develop chronic HBV infection (Chisari et al., 2010; Das et al., 2010). However, a recent study suggests that induction of effective CD8 T cell specific immune response to HBV is dependent on early CD4 T cell priming which might be regulated by the size of viral inoculum (Asabe et al., 2009). In acute hepatitis infection HBV is detectable

already in the incubation period. Interferons up regulate the expression of major histocompatibility complex (MHC) class I antigens on the cell surface which allows detection and elimination of infected cells.

The MHC class II restricted immune response is foremost directed to the capsid proteins (HBcAg and HBeAg). The immune response to HBsAg is lower than to capsid proteins. B-cells response to HBcAg and to a later time point to the large, the middle and the small surface proteins. Neutralizing antibodies (anti-HBs) to the virus block the spread of HBV and infection of hepatocytes. These antibodies to HBsAg also protect from reinfection with the virus (Modrow et al., 2003).
The chronic HBV infection occurs, mainly, due to the lack of HBV specific adaptive T-cell immune response and lack of HBsAg specific humoral immune response.

1.6. Diagnosis of acute and chronic HBV infection

The diagnosis of HBV infection is based on clinical, biochemical, histological and serologic findings. The interpretation of these findings is essential for the diagnosis of the various clinical forms of HBV infection. Table 2 gives an overview of serological and virological markers and their interpretation (Liang, 2009).

Table 2: Hepatitis B virus serological and virological markers

Marker	HBV marker for:
HBsAg	HBV infection (acute and chronic)
HBeAg	High level HBV replication and infectivity (also marker for treatment response)
HBV DNA	Level of HBV replication (also primary marker for treatment response)
Anti-HBc (IgM)	Acute HBV infection (sometimes also in flare of chronic infection)
Anti-HBc (IgG)	Recovered or chronic HBV infection
Anti-HBs	Recovered HBV infection and marker of HBV vaccination (Titer level reflects vaccine efficacy)
Anti-HBe	Low-level HBV replication and infectivity (also marker for treatment response)
Anti-HBc IgG and (and no) anti-HBs **and HBsAg neg**	Recovered HBV infection
Anti-HBc (IgG) and HBsAG **(> 6 Months)**	Chronic HBV infection
Anti-HBc (IgG) and/or anti-HBs and HBV-DNA	Latent or occult HBV infection

The typical course of serological markers in acute HBV infection is shown in figure 4A. HBsAg is the first serological marker of acute HBV infection and is generally detectable after infection from week six on. From week eight on anti-HBc-IgM and HBeAg are almost present. IgG antibodies against HBcAg can first be detected after the peak of viraemia and at the time point of increasing transaminases. The first diagnostic parameters of the recovery from infection are reduction of viral load and short afterwards reduction of HBsAg and HBeAg in peripheral blood (Gerlich et al., 2009).

The chronic HBV infection starts with similar serological pattern as the acute disease (Fig. 4). However, chronically infected patients do not lose HBV DNA, and rarely HBsAg and HBeAg. HBsAg is useful for diagnosis of transition from acute to chronic infection. Persistence of HBsAg over six months confirms the diagnosis of chronic HBV infection. The HBV DNA is a marker of active HBV replication in acute and chronic HBV infection. The amount of HBV DNA detected by quantitative PCR is an indicator of infectiousness. Anti-HBc is a good parameter to determine the infestation rate of HBV in a population. So far HBcAg is not used as part of a vaccine and is only detectable after infection with HBV.

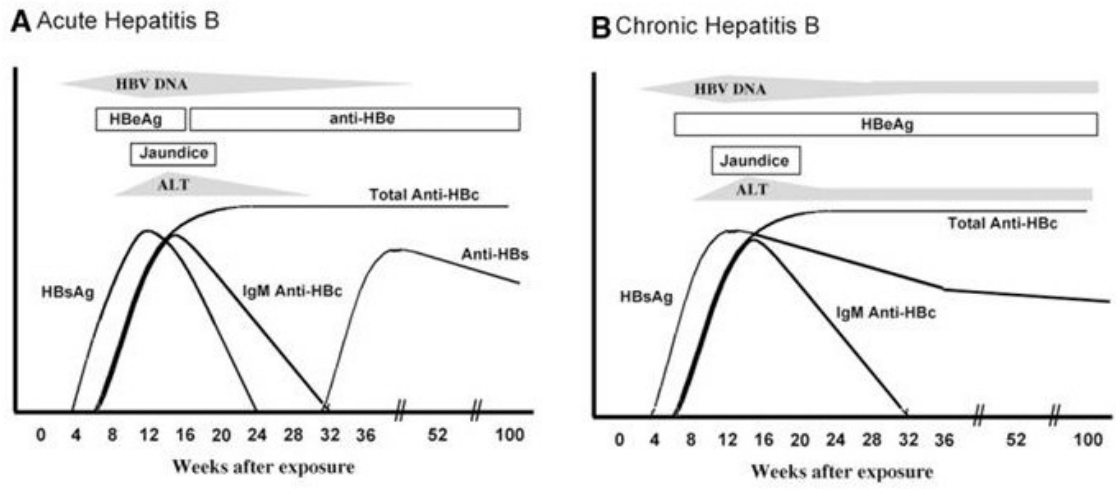

Figure 4: Clinical course and serological profiles of acute (A) and chronic (B) HBV-infection. Figure according to Liang (Liang, 2009).

1.7. Therapy of chronic HBV infection

Chronically HBV infected patients belong to a risk group to develop liver cirrhosis and in sequel hepatocellular carcinoma (HCC). High load of HBV DNA is associated with development of cirrhosis and HCC. HCC occurs predominantly in countries with a high prevalence of HBV. The first therapy of chronic HBV infection is treatment with interferon-alpha. Particularly, the pegylated form of interferon-alpha acts as deposit and is the preferred

form for treatment. 30% of the chronic infections can be treated successfully with this medication. Therefore therapy with peg-IFN has become the first-line treatment (Takkenberg et al., 2010).

Further, treatment with nucleoside analogues (NUCs) has been currently approved. The most frequently used nucleoside analogue in the past was lamivudin (3TC; 2',3'-Dideoxy-3'-Thiacytidin). Lamivudin is an inhibitor of pyrophosphorolyse activity of the viral reverse transcriptase and is used and developed for treatment of HIV infection. In contrast to retroviral infection the HBV pregenome RNA is transcribed into the DNA before secretion of the virions into the plasma. Due to the half-life of particles in the plasma of about 1.5 days, the reduction of the viral load can be reached very fast after medication. However, medication with NUCs cannot eliminate the virus due to the persistence of HBV cccDNA in the nucleus of hepatocytes. Further, only 10 to 20% of patients lose HBeAg one year after therapy. The same proportion of patients develops resistance to lamivudin due to mutation in the YMDD motif of the HBV polymerase gene and therefore a viral rebound occurs. The emergence of resistance to lamivudin led to development of new less resistance susceptible NUCs like entecavir and tenofovir (Tab. 3) (Schaefer et al., 2009). Tenofovir and entecavir have a markedly lower risk of resistance than the other approved NUCs, therefore these drugs are preferentially used as a first-line therapy today.

Table 3: Approved nucleosid analogues (NUCs) for HBV treatment

Drug	Structure
Lamivudine	Cytidine analogue
Adefovir	Adenosine analogue
Entecavir	Guanosine analogue
Telbivudine	Thymidine analogue
Tenofovir	Adenosine analogue

A final therapeutic option for patients with carcinoma and liver failure due to HBV is liver transplantation. The therapy of chronic HBV infected patients after liver transplantation is difficult due to immunosuppressive medication. However, these patients have to be treated with NUCs and immunoglobulins directed against HBsAg. The reinfection of the new liver

can nevertheless occur due to circulating infectious virus particles in the peripheral blood (Lai et al., 1998; Tillmann et al., 1999).

1.8. Vaccines against Hepatitis B

In the 1980s spherical and filamentous HBsAg particles purified from plasma of HBsAg carriers were used as a vaccine against HBV (Maupas et al., 1976). The plasma of the chronic carriers was inactivated to avoid the potential risk of residual infectious HBV, HIV particles or infection with other pathogens. The vaccine consisted of 22 nm HBsAg particles.

Second generation vaccines are recombinant non-glycosylated small surface proteins (S) produced in yeast (McAleer et al., 1984). These vaccines have been shown to be highly immunogenic and are used in vaccination programs in more than 150 countries worldwide. However, approximately 4-8% of vaccinees in the first three decades of life, and over 10% in the fourth decade and onwards do not seroconvert following immunization (McAleer et al., 1984; Andre, 1990).

Recently, third generation vaccines containing not only the S protein, but also the middle (M) and the large (L) surface proteins has been developed (Hourvitz et al., 1996). These vaccines, produced in mammalian Chinese hamster ovary (CHO) cells, have been shown to induce an immune response, which occurs earlier and is stronger than that induced by S alone (Raz et al., 1996). Even in many so called “non-responders” to S protein a protective immune response was induced (Rendi-Wagner et al., 2006).

1.9. Future vaccines

In the present the majority of persons vaccinated against hepatitis B successfully respond to vaccination, however approx. 5% of persons are non-responders. This situation clearly emphasizes the need of improved prophylactic preparations that would prevent spread of HBV. Simultaneously, new optimized preparations have to be examined for their sufficiency in therapeutic vaccination of individuals suffering from chronic hepatitis B infection. The preS1 region of HBV large surface antigen is involved in attachment and entry of HBV into hepatocytes (Fig. 5). Immunization of mice with PreS1 induced antibodies, which neutralize HBV in tissue culture (Glebe et al., 2005). Therefore, inclusion of preS1 sequence of the hepatitis B virus (HBV) envelope into immunogens should improve the efficacy of the vaccination, inducing a wider spectrum of antiviral antibodies. In support of this, it has been shown that anti-PreS antibodies appear early during HBV infection, before the detection of anti-hepatitis B surface antibodies (anti-HBs) (Neurath et al., 1986a; Neurath et al., 1986b; Le

Seyec et al., 1999; Maeng et al., 2000). Their appearance is usually followed by recovery from HBV infection. In contrast, anti-PreS antibodies are exceptionally rare in chronic hepatitis B (Neurath et al., 1986a; Maeng et al., 2000; Zhang et al., 2011b).

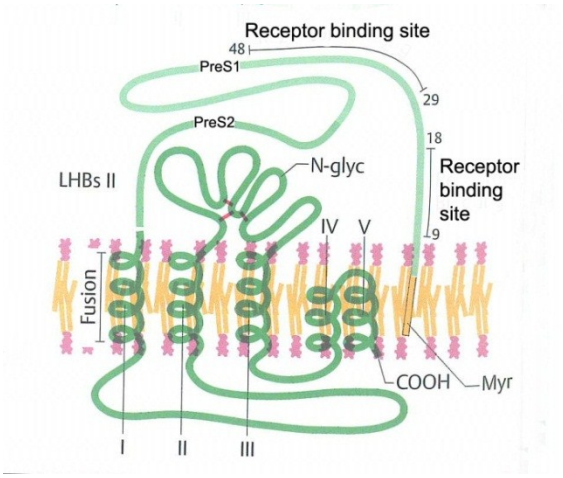

Figure 5: Topologic model of HBsAg at the endoplasmic reticulum membrane.
The binding site of PreS1 region into hepatocytes is indicated. Figure according to Gerlich (Gerlich et al., 2009).

In the past, a new type of vaccines, which uses virus like particles (Fig. 6), was developed. One of such vaccines is the highly immunogenic HBV core protein (HBcAg) a very potent protein for induction of cellular immune response against HBV (Borisova et al., 1990).

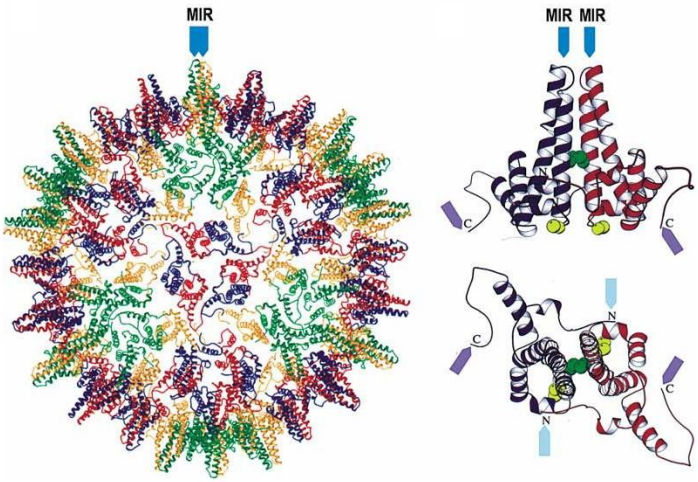

Figure 6: Topologic model of HBV core particle.
The particle (left) consists of 120 core protein dimers (right). The major immunodominant region (MIR) is indicated. Figure according to Pumpens (Pumpens et al., 1995).

However, antibodies to HBcAg do not protect against HBV infection (Ferrari et al., 1988; Jung et al., 1995).

This highly immunogenic HBcAg particle (120 core dimers = 1 core particle) can be used as a carrier of foreign B- and T-cell epitopes to enhance the immune response (Milich et al., 1997a; Milich et al., 1997b). Therefore, the main advantage of chimeric HBcAg particles is the induction of high titres of antibodies and simultaneously vigorous T-cell mediated immune responses (Pumpens et al., 2001). Therefore such particles are a promising approach for creation of a new type of HBV-specific immunogen.

In the past the PreS1 fragment of HBsAg, which is able to induce virus-neutralizing antibodies (Deepen et al., 1990; Pizarro et al., 2001; Glebe et al., 2003), was inserted into the HBcAg particle (Lachmann et al., 1999). The resulting chimeric virus like particles (VLPs) containing PreS1 increased the immune response to HBV in mice. In these experiments, however, the ability of the chimeric particles to provide protection against a challenge with HBV was not examined.

1.10. Animal models in Hepatitis B research

Chimpanzees are the only animals fully permissive for HBV infection. Conversely, their use for research purpose is severely limited by the high costs and strong ethical constrains. In general, most of progresses in hepatitis B virus research are based on infection studies with HBV-related animal models. The Woodchuck and its Woodchuck hepatitis virus (WHV) infection serves as an important model for pathogenesis and immune reactions caused by HBV in humans (Roggendorf et al., 2007; Roggendorf et al., 2010). Most preclinical testing of antiviral treatment against HBV is done with WHV-infected woodchucks (Lu et al., 2008). WHV and HBV are members of the virus family Hepadnaviridae (Lu et al., 2001) which cause persistent infections of hepatocytes, persistent viremia, and antigenemia.

The genome of WHV is, similar to HBV, circular and partial double stranded with a length of approximately 3.3 kb. The WHV envelope proteins also consist of the small, the large, and the middle proteins. The WHV genome is encapsulated by the nucleocapsid (core) protein (WHcAg). Similar to HBV core the WHV core was already shown to induce a strong humoral and cellular immune response. Furthermore, protection against the challenge was observed in woodchucks immunized with WHcAg (Menne et al., 1997; Menne et al., 1998). Recently, VLPs based on the WHcAg were described (Billaud et al., 2005a).

However, neutralizing antibodies to the PreS1 epitopes of the WHV were still not examined. If a protective effect is demonstrated, this type of chimeric VLPs might be considered as a prototype of a new HBV prophylactic and/or therapeutic vaccine for the use in humans.

1.11. Transfer of cells of the immune system by transplantation

In 1942 Landsteiner et al. reported for the first time that specific immunity was transferred from one patient to another by lymphocyte transmission (Landsteiner, 1942). From that time on, adoptive immune transfer was described by transfer of lymphocytes from mesenteric lymph node cells, gut intraepithelial lymphocytes, peritoneal cells, spleen cells and bone marrow cells (Ghadirian et al., 1983; Iverson et al., 1983; McDonald et al., 1996).

In recent years studies show successful adoptive transfer of immunity against infectious diseases, like HBV in bone marrow transplant patients (Peters et al., 2000; Kupeli et al., 2002; Serap et al., 2010). In 1995 Shouval et al. systematically analysed adoptive transfer in mice in which immunity against HBV was transferred by bone marrow transplantation from the donor to the recipient (Shouval et al., 1995). The same group showed in an initial clinical study (Ilan et al., 1993) that 12 bone marrow transplanted recipients who were HBV-carriers recovered from viral infection after receiving bone marrow from healthy donors immune to HBV (anti-HBc+/anti-HBs+). Before transplantation the recipients had been anti-HBs negative. Post-transplantation they developed protective anti-HBs levels of above 10 mIU/ml antibodies. The patients remained protected from HBV and none of them suffered from acute HBV infection during the follow-up period of 7 months. An immune transfer via hematopoietic stem cell transplantation could also be observed after electively vaccinating donors against HBV (Lindemann et al., 2003a). Dahmen and co-workers systematically analysed the adoptive transfer of immunity against the hepadnaviral infection by liver transplantation in the rat and the woodchuck model (Dahmen et al., 2003a; Dahmen et al., 2003b; Dahmen et al., 2004). Results of this study showed, that the transfer of immunity from donor to recipient by liver transplantation is feasible.

1.12. Aim of the thesis

Liver transplantation is often the ultimate option of therapy for patients with cirrhosis due to chronic hepatitis B virus (HBV) infection. Due to organ shortage the living liver donation between relatives increased steadily in the past and represents one cohort of liver donation. However, prevention of reinfection with HBV by HBV Immunoglobulin is cost-intensive and in several cases not effective.

The transfer of HBV immunity in rats after liver transplantation and subsequent experiments in woodchucks showed the suppression of severe reinfection in chronically infected liver recipient animals after transplantation of the liver from uninfected animals to chronically WHV infected animals.

These findings encouraged us to examine the transfer of immunity to HBV from living donor to a chronically HBV infected recipient. Donors and recipients undergo a transplantation evaluation program within 1-2 months. However, this time is too short for inducing suitable immune response to HBV with a standard vaccination scheme (6 months).

Therefore, the first aim of this study was to establish a short time immunization protocol to induce high HBV specific B and T-cell response with a new vaccine which contains PreS1, PreS2 and S protein in healthy volunteers within a short period of 1-2 months.

The second aim was to immunize living liver donors using the short time immunization protocol and to examine the transfer of immunity from immunized donors to HBV negative recipients and chronic HBV infected recipients after transplantation.

The aim of the third part of this thesis was to improve a current vaccine to HBV by including PreS1 protein of HBV to the vaccine which induces high titer of antibodies to PreS1 which inhibits binding of HBV to its receptor NTCP. Therefore virus like particles (VLPs) composed of the WHV core protein with insertions of PreS1-derived peptides should be generated. The humoral immune response against the VLPs should be examined in mice and protection can be tested in the preclinical animal model (woodchuck).

2. Materials and Methods

2.1. Study Population

2.1.1. Patients

This study was approved by the institutional review board (AZ: 03-2234).
For evaluation of the short time immunization protocol 30 volunteers were immunized against HBV. All 30 volunteers provided written informed consent.
46 living liver donors were immunized against HBV and 14 liver recipients received the liver from an immunized donor. All 46 living liver donors and 14 liver recipients provided written informed consent. All vaccinations in humans were performed by Melanie Fiedler and Monika Lindemann.

2.1.2. Animals

For immunization experiments BALB/cJ (H-2d) mice were used. They were kept under standard pathogen-free conditions in the Institute of Virology, University Duisburg-Essen. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011) and were reviewed and approved by the local Animal Care and Use Committee (Animal Care Center, University of Duisburg-Essen, Essen, Germany), and the district government of Düsseldorf, Germany. For immunization and taking blood mice were anesthetized with Isoflurane.

2.2. Chemicals

All chemicals, unless stated otherwise, were purchased from Gibco (Karlsruhe, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Sigma (München, Germany).

2.2.1. Commercial kits and reagents

Qiagen Plasmid Mini Kit	(Qiagen, Germany)
Qiagen Plasmid Midi Kit	(Qiagen, Germany)
QiaQuick Gel Extraction Kit	(Qiagen, Germany)
TOPO™ TA Cloning® Kit (Version R)	(Invitrogen, Germany)
Taq DNA Polymerase (5U/µl)	(Promega GmbH, Germany)
10x Reaction buffer	(Promega GmbH, Germany)
$MgCl_2$ (25mM)	(Promega GmbH, Germany)

dNTP (10mM)	(Promega GmbH, Germany)
100 bp-DNA-Ladder plus	(Fermentas, Germany)
M-MLV RT 5x Reaction buffer	(Promega GmbH, Germany)
M-MLV Reverse Transcriptase (200U/µl)	(Promega GmbH, Germany)

2.2.2. Cell culture conditions

Human PBMCs were cultured in RPMI 1640 medium. Before usage 10% human serum (separated from pooled sera of blood donors), 10 mg/ml penicillin/streptomycin (PAA Laboratories, Austria) and 2mM L-glutamine were added. Mice cells isolated from spleen were cultured in RPMI 1640 medium. Before usage 10% fetal serum albumin, 10 mg/ml penicillin/streptomycin (PAA Laboratories, Austria) and 2mM L-glutamine were added.

2.2.3. Peptides and proteins

The HBV PreS1 peptide (aa 1-108) and the HBV S-protein (227 aa) were purchased from Acris Antibodies (Hiddenhausen, Germany). The HBV S, M and L protein containing suspension (produced in CHO cells) was kindly provided by Berna Biotech (Bern, Switzerland). The WHV PreS1 peptide (aa 1-81) was a gift from Stephan Urban, Heidelberg.

2.2.4. Plasmids

WHV core particles were expressed using the PQE60 plasmid from Qiagen (Qiagen, Germany). Plasmids for PreS1-GST transformation and expression were purchased from Invitrogen (see table 4) using E. coli Expression System with the Gateway® Technology. Schematic maps of the plasmids were included into the appendix (see section 7.1).

Table 4: Plasmids

Plasmid	gene of resistance	length (bp)	Company
PQE-60	ampicilin	3431	Qiagen
pENTR™/SD/D-TOPO®	kanamycin	2601	Invitrogen
pDEST24™	ampicilin	6961	Invitrogen
pCR®2.1-TOPO®	ampicilin	3931	Invitrogen

2.2.5. Bacterial strains

Bacterial strain E. coli JM 109 was used for transformation and expansion of the plasmid PQE60 and BL 21 for protein expression from the plasmid PQE60. For transformation of pENTR™/SD/D-TOPO® plasmid strain TOP 10 One Shot™ (Invitrogen, Germany) cells were used. The transformation of pDEST™ was performed using the bacterial strain DH5α™. For PreS1-GST expression E. coli strain BL21-AI™ One ShotR was used. Table 5 summarizes the genotype of all used bacterial strains.

Table 5: Bacterial strains

E. coli strain	Genotype
JM 109	*rec*A1 *sap*E44 *end*A1 *hsd*R17*gur*A96 *rel*A1 *thi*(*lac-pro*AB) F´[*tra*D36*pro*AB+*lac*Iq*lac*ZΔM15]
BL 21	F- *ompT hsdS*B (rB-mB-) *gal dcm*
TOP 10 One Shot™	F´*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 ΔlacX74 *deo*R *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G
DH5α ™	F- *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1
BL21-AI™ One ShotR	F- *ompT hsdS*B (rB-mB-) *gal dcm araB*::*T7RNAP-tetA*

2.2.6. Restriction enzymes

Table 6 summarizes all enzymes, which were used for restriction of PCR products or/and for restriction of the plasmids.

Table 6: Enzymes

Enzyme	Restriction type
NcoI	5' C^CATGG 3' 3' GGTAC^C 5'
BglII	5' A^GATCT 3' 3' TCTAG^A 5'
Bsp119I	5' TT^CGAA 3' 3' AAGC^TT 5'

2.2.7. PCR primers

Table 7 summarizes all primers which were used in the PCR.

Table 7: PCR-Primer

Primer	Sequence
Pep1	Forward: 5´-ctt cga aaa tgg gca aca aca taa aag-3´
	Reverse: 5´-tat ttt cga aca ctg act ggt tct gag-3´
Pep2	Forward: 5´-ctt cga aat att aca caa cca ctt acc-3´
	Reverse: 5´-tat ttt cga ata taa gaa cag agt cca-3´
Pep3	Forward: 5´-ctt cga aaa atc cca aaa atc aac aag aa-3´
	Reverse: 5´-tat ttt cga ata att ttt gat cca cag g-3´
Pep4	Forward: 5´-ctt cga aaa ctt ggc aag gat ttc ctg t-3´
	Reverse: 5´-tat ttt gta ctt tga ttt cga aag ttt gag c-3´
Pep5	Forward: 5´-ctt cga aaa tca aac ctg ggc cta taa-3´
	Reverse: 5´-tat ttt cga acc ctt gat ctc gat ttg t-3´
Pep6	Forward: 5´-ctt cga aac cgc ctc aaa ctc caa caa-3´
	Reverse: 5´-tat ttt cga aag tta agt ggg ggt gag tat-3´
PreS1 pDest	Forward: 5´-cac cat ggg caa caa cat aa-3´
	Reverse: 5´-ggt taa gtg ggg gtg agt at-3´
6DC	Forward: 5'-gca cac gga att ccg agg act ggg gac cct g-3'
S7D	Reverse: 5'-gac acc aag ctt ggt tag ggt tta aat gta tac c-3'

2.2.8. Instruments

Instrument	Manufacturer
Autoclave	Lautenschläger, Germany
Cell culture incubator	Heraeus, Germany
Gel electrophoresis box	Medizintechnik, University Hospital Essen, Germany
Electrophoresis PowerPack P25	Biometra, Germany
ELISA-Reader	SorinBiomedica, Italy
Bioreader 2000	Biosys, Germany
Imaging System GeneGeniusBio	Synoptics, United Kingdom
UV transluminator FLX-20M	MWG-Biotech, Germany
Light microscope	Carl Zeiss AG, Germany
Freezer -20 °C	Liebherr, Germany
Cell culture hood HeraSafe	Heraeus, Germany
Microwave	Panasonic, Germany
Thermocycler Mastercycler gradient	Eppendorf, Germany
Thermocycler PxE0.5 Thermal Cycler	ThermoScientific, USA
LightCycler 2.0	Roche, Germany
PH meter MP 220 pH Meter	Mettler Toledo, Germany

Spectrophotometer Gene Quant pro	Amersham Bioscience, USA
Pipettes	Eppendorf, Germany; Gilson, USA
Vortex Genie2	Scientific Industries, USA
Scales	Sartorius AG, German
Water bath	Julabo, Germany
Centrifuge 5415C, 5810	Eppendorf, Germany
Megafuge 1.0R	Heraeus, Germany

2.2.9. Vaccines against Hepatitis B

Healthy volunteers (n=15) and living liver donors (LLD) were vaccinated using Sci-B-Vac (kindly provided by SciGen Ltd., Singapore, Singapore). The standard vaccine HBVAXPRO® (Sanofi Pasteur MSD, Frankfurt, Germany), containing only S protein, was used for vaccination of healthy volunteers (n=15) and of living liver donors with persisting vaccine derived immune response against HBV. The distribution of L, M and S Protein in Sci-B-Vac and HBVAXPRO® vaccine is shown in figure 7.

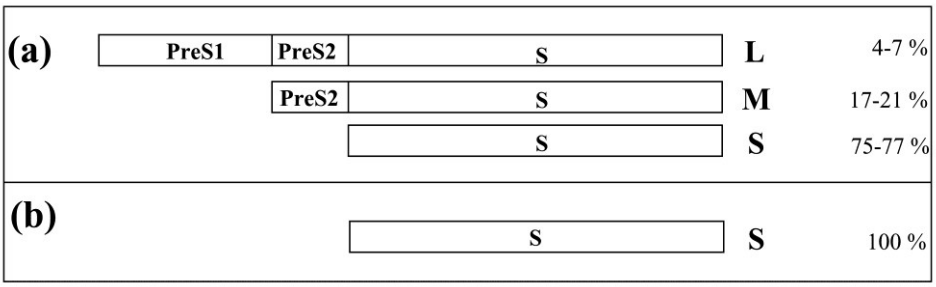

Figure 7: Distribution of PreS1, PreS2 and S protein in Sci-B-Vac and HBVAXPRO® vaccine.
(a) Distribution of PreS1, PreS2 and S protein in Sci-B-Vac vaccine produced in Chinese hamster ovary cells (CHO). (b) S protein in yeast derived HBVAXPRO® vaccine.

2.3. Methods

2.3.1. Immunization of healthy volunteers

In total 30 healthy volunteers were randomly immunized either with 20 µg Sci-B-Vac (n = 15; female: 8, male: 7) or a German standard HBV vaccine HBVAXPRO® at the recommended dose of 10 µg (n = 15; female: 9, male: 6) four times at two-week intervals. We examined the humoral (anti-HBs titer) and cellular (ELISpot, proliferation assay) immune responses before each administration, four weeks after the last immunization, and again one year after immunization.

2.3.2. Immunization of living liver donors

Potential LLD (n = 46) received immunizations either with Sci-B-Vac (n = 39), a third generation vaccine up to 5 times at least biweekly with a 20 µg dose or booster immunizations with HBVAXPRO® (n = 7), a second generation vaccine at the recommended dose of 10 µg. Humoral and cellular immune responses in donors were determined by anti-HBs titer, proliferation assay, and IFN-γ ELISpot after immunization and in LLD also before transplantation.

2.3.3. Treatment and monitoring of immune responses in recipients

In recipients (n = 14) HBV specific humoral and cellular immune responses were measured prior to and after transplantation at least monthly by the same assays. To prevent rejection of the graft, recipients were treated with different immunosuppressive drugs. Usually cyclosporin A or tacrolimus, mycophenolate mofetil and prednisone were administered. The reduction of immunosuppressive medication differed due to the recipient's requirements. Chronically HBV infected patients received, in addition, passive immunoprophylaxis using Hepatitis B specific immunoglobulin (HBIg Hepatect, Biotest, Dreieich, Germany). HBIg was administered intravenously using a previously described protocol (Beckebaum et al., 2003). Combination of this medication with the nucleoside analogue lamivudine was indicated, when HBV DNA was detectable after transplantation.

2.3.4. Immunization of mice

BALB/cJ mice aged 6–8 weeks were immunized subcutaneously using 20 µg of either WHcPep3, WHcPep4, WHcPep6 and WHV PreS1 peptide (aa 1-81), each vaccine emulsified in incomplete Freund's adjuvant (IFA; Sigma, Deisenhofen, Germany). Mice received three immunizations in a time interval of two weeks and were sacrificed three weeks after the last immunization for determination of serum antibodies (1:100 dilution) against WHV PreS1. Cellular immune response was measured using WHV PreS1-GST protein for stimulation in a proliferation assay.

2.3.5. Proliferation assay with human PBMCs

The proliferation assay was performed in round bottom microtiter plates with 2.5 x 10^5 PBMCs per 200 µl culture by stimulation with the same antigen concentrations, which were already described for the ELISpot assay. Cell culture conditions and measurement of [3H]

thymidine uptake followed a protocol described previously (Lindemann et al., 2002). For the evaluation of proliferation assays median values of triplicate cultures were considered. The stimulation index (SI) was defined as the quotient of HBV-specific and autologous (unstimulated) proliferative responses. Values of SI > 2.5 were defined as positive as described before (Lindemann et al., 2002).

2.3.6. Interferon-γ ELISpot

PBMCs from heparinized blood were separated by Ficoll–Hypaque™ (Amersham Pharmacia Biotech, Sweden) density gradient centrifugation. 4 x 10^5 PBMCs in 200 µl culture volume were pre-incubated with 1 µg/ml S, M and L protein, or 1 µg/ml L protein fragment (108 aa), or 0.5 µg/ml of the small S protein (227 aa) in round bottom microtiter plates (Becton Dickinson Labware, Germany). After 48 h the cells were transferred from round bottom microtiter plates to pretreated ELISpot plates.

Pretreatment of ELISpot plates: Wells of the MultiScreen-HA ELISpot plates (MAHAN4550, Millipore, Bedford, MA, USA) were coated for 2 h with antibody specific for human IFN-γ at 37°C or overnight at 4°C. Thereafter, plates were washed 3 times with PBS and blocked for 1 h at room temperature with 10% human serum in RPMI-medium.

Detection of IFN-γ producing cell clones: After 48 h cells were discarded; plates were washed 6 times with 0.05% Tween 20 containing PBS solution and incubated for 1 h with human IFN-γ biotinylated second antibody. Subsequent cytokine production of IFN-γ was detected by a solid phase ELISA as described previously (Lindemann et al., 2003b). Numbers of spots were analyzed by the Bioreader 2000 (Biosys, Germany). For the evaluation of IFN-γ production median values of triplicate cultures were considered. ELISpot results were generated as HBV-specific minus the autologous (unstimulated) values. Negative values were set as zero. Before the first immunization cellular immune responses following stimulation with HBV antigens were measured. The mean value plus the sum of three standard deviations (SD) was set as threshold to define unspecific IFN-γ production. An ELISpot response among the immunized groups was considered positive if a value of spots ≥ 9 was achieved after PreS1 (aa 1-108) stimulation (value of spots ≥ 10 after S, M, L and of spots ≥ 16 after S stimulation).

2.3.7. HBV specific serology

Serum was separated by centrifugation and HBsAg, anti-HBc, HBeAg, and anti-HBs were measured by Chemiluminescent Microparticle Immunoassay (Architect System, Abbott Laboratories, Wiesbaden, Germany) following the manufacturer's instructions. The detection limit of the anti-HBs titer is 10 IU/l.

2.3.8. WHV specific serology

Serum was separated by centrifugation and anti-WHV PreS1 antibodies of mice sera were detected by enzyme-linked immunosorbent assay (ELISA). Therefore MicroELISA plates (Maxisorp, Nunc, Germany) were coated with 150 ng of the PreS1-GST protein per well in 50 µl 0.1 M sodium carbonate buffer (pH 9.5) at 4°C. Mice sera were diluted 1:100 in loading buffer (PBS supplemented with 3% BSA and 2% Tween 20) and added to the PreS1-GST protein-coated wells. The antibodies were incubated for 2 h at 37°C followed by four washes with 0.05% Tween 20 in PBS. Bound antibodies were detected using HRP-conjugated anti-mouse IgG antibodies (cat. no. 02067E, PharMingen, Germany) at a dilution of 1:2000 followed by incubation with o-phenylendiamine and 2x HCl (cat. no. 6172-24, Abbott, Germany) in PBS (pH 6.0). The reaction was stopped by 1 M H_2SO_4 and the extinction was determined at 492 nm.

2.3.9. Administration of Immunoglobulin to HBsAg (HBIg)

Patient 1 received 1,000 IU anti-HBs (HBIg) per day intravenously for 21 days. These antibodies were diluted in approximately 3.5 l plasma of a 75 kg heavy recipient and resulted in an anti-HBs titer of 286 IU/l. This value could only be an assumption to the real titer found in individual patients; however, it seems to be accurate enough to roughly calculate the expected HBIg derived anti-HBs titers. The portion of the HBIg derived antibody titer was calculated considering an anti-HBs half-life of 21 days (Partovi et al., 2001) and using generally approved formulas (Birkett, 2002). Additionally, the recipients required fresh frozen plasma during the surgical treatment that contained small amounts of anti-HBs. Therefore, plasma derived anti-HBs was measured and the expected anti-HBs titer decay was calculated as described above.

2.3.10. Qualitative and quantitative detection of HBV DNA

HBV DNA was extracted from 200 µl of serum using QIAamp blood kit (Qiagen, Germany). The qualitative HBV DNA detection was performed with the Artus HBV LC PCR Kit (Qiagen, Germany) using the LightCycler 2.0 (Roche Diagnostics GmbH, Germany) following the manufacturer's instructions. Here, the detection limit is 100 IU/ml. The quantification was performed with the VERSANT HBV DNA 3.0 Assay (bDNA) (Siemens, Germany) on the BAYER VERSANT 440 MOLECULAR SYSTEM (Siemens, Germany) following the manufacturer's instructions. The detection limit of HBV DNA is 357 IU/ml.

2.3.11. Determination of HBV subtype by PCR

HBV DNA was extracted from serum as already described and subjected to PCR amplification for the HBV genome region comprising the s gene. Ten µl of extracted DNA were amplified in a 50 µl reaction mixture with the primers 6DC (nt 131-148) and S7D (nt 845-825). The nucleotide positions of primers are numbered according to a published complete HBV sequence (GenBank accession number AY220698). The PCR product was cloned into pCR®2.1-TOPO vector (Invitrogen, Germany) and subjected to DNA analysis. The subtype of HBV isolates was determined according to the HBsAg sequence.

2.3.12. Computer based structure prediction

3D-JIGSAW is an automated system based on the analysis of homologues of known structures and used to build three-dimensional models of proteins (Bates et al., 2001). This software was applied to identify the MIR of the WHcAg. The structure prediction was made by comparison with the known structure of the HBV core protein (PDB-ID: 1QGT). The protein was visualized using the UCSF Chimera package (Pettersen et al., 2004) from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).

2.3.13. Construction of vectors containing core and preS1 epitopes

The pQE60 vector was used for the expression of the truncated WHcAg (149 aa residues) (NCBI accession no. J04514) inserted between the NcoI-BglII restriction sites (kindly provided by Menji Lu, Essen, Germany). The Bsp119 insertion site between the amino acids 78 and 79 of the truncated WHcAg (149 aa residues) was kindly provided by Melanie Fiedler, Essen, Germany. Six overlapping fragments of the PreS1 protein (149 aa in length) gene were

generated by PCR using WHsAg (NCBI accession no. M19183) gene sequence as a template, and six preS1 specific pairs of primers (see table 7: Pep1-Pep6), which additionally included the Bsp119 restriction site. Table 8 summarizes the PCR conditions for PCR products PCRPep1-PCRPep6.

Table 8: PCR conditions for PCR products PCRPep1-PCRPep6

Cycle	Temperature (°C)	Time (min)	Cycle-number
Denaturation	94	4	1
Denaturation	94	1	
Annealing	60	1	35
Elongation	72	2	
Delay	72	7	1

Each resulting PCR product (PCRPep1-PCRPep6) and the PQE60 vector containing the truncated WHcAg gene sequence modified with Bsp119 restriction site (Q60WHcBsp) were digested by the Bsp119 restriction enzyme and ligated with the T4 DNA ligase to create six new WHcAg constructs with the corresponding insertions. The correct reading frame of the inserts was verified by sequencing. The propagation and the purification of the plasmids (Qiagen, Germany) and the ligation reactions (Fermentas, Germany) were performed using commercial kits according to the manufactures instructions (Qiagen, Germany and Fermentas, Germany). The transformation of the plasmids into the BL21 E. coli strain allowed the expression of the corresponding protein under the control of the T5/Lac operon promoter.

2.3.14. Protein expression, purification and capsid assembly

E. coli was grown on a rotary shaker at 37°C until an optical density of 0.6-0.8 at OD540 was reached. The expression of the protein was induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranosidase) and additional incubation for 3 h. Cells were pelleted and lysed using the lysozyme-salt solution, aluminium oxide or sonication, respectively.

The gentle lysozyme salt solution lysis was performed by dissolving the cells in one volume of the lysis buffer 1 (45 mM Tris/HCl pH 8, 4.5 mM EDTA pH 8, 0.45 µM phenylmethylsulfonylfluorid (PMSF)), two volumes of lysis buffer 2 (2 mg lysozyme per ml buffer 1) and one volume of lysis buffer 3 (0.4% Triton X-100 in buffer 1). Afterwards, the suspension was incubated for 10 min at -80°C and thawed in the cold. This step was repeated three times following addition of 1/6 volumes of DNase buffer (2 mg DNAse per ml 1 M $MgCl_2$) and incubation for 30 min at 4°C.

The gentle method of cell disruption was performed by adding of 1 volume aluminium oxide powder to the frozen cells following crushing in a bowl for 30 min at 4°C. After addition of 3 volumes of buffer 1 and 1/12 volumes of the DNAse buffer the suspension was incubated for 30 min at 4°C. Sonication, the vigorous method of cell disruption was performed by addition of three volumes of buffer 1 and one volume of buffer 3 to the cells following 5 x 15 sec sonication (35 kHz) with a 45 sec distance between each.

The protein was removed from debris by washing the pellet with PBS buffer or PBS buffer containing 0.45 M urea or 1.5 M urea. The resulting supernatant was precipitated overnight (4°C) with 20 to 33% of ammonium sulphate. The precipitated protein was recovered by washing with PBS buffer containing 0.1% Triton X-100 or Triton X-100 containing 0.45 to 1.5 M urea. 5-7 ml of the supernatant were loaded on a 20-60% sucrose gradient and centrifuged overnight at 27,000 rpm, or on a Sepharose CL4B column, respectively, and eluted with PBS buffer without Triton X-100. The presence of WHV core protein in 2 ml fractions was tested by polyacrylamid gel electrophoresis (PAGE). Positive fractions were pooled and concentrated by ammonium sulphate precipitation overnight at 4°C as described above. Pellets were dissolved in PBS buffer or PBS buffer containing 0.45 M urea, respectively. The supernatant was dialyzed against 2000 volumes of PBS buffer and stored at -20°C in 50% glycerol or 50% sucrose, respectively.

2.3.15. PAGE and Western blotting

For the polyacrylamide gel electrophoresis (PAGE) bacteria were pelleted and the supernatant and the debris samples after lysis were suspended in the SDS-gel electrophoresis sample buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.1 mg/ml bromphenol blue). After heating at 100°C for 10 min the proteins were separated by Laemmli`s polyacrylamide gel electrophoresis with 15% running gel.

Acrylamide stock:	29.1 g acrylamide; 0.9 g NN-methylenbisacrylamide; Add water to 100 ml
Loading-gel:	0.8 ml acrylamid stock; 3.6 ml water; 0.5 ml 1.875 M Tris-HCl pH 8.8; 50 µl 10% Sodium dodecyl sulfate (SDS); 5 µl Tetramethylethylenediamine (TEMED); 17 µl Ammonium persulfate (APS)
Separating-Gel (15% SDS):	7.5 ml Acrylamid stock; 4.3 ml water; 3 ml 1,875 M; Tris-HCl pH 8,8; 150 µl 10% SDS; 7.5 µl TEMED; 50 µl APS

Western blot was performed by transfer of the proteins into the nitrocellulose sheets (0.2 µm, Millipore, USA) and following incubation with anti-HBc mAb 14E11 (1:1,000), which recognizes HBcAg aa residues 136-144 (Skrivelis et al., 1993) and the WHcAg as well. After incubation for 1 h at room temperature the sheets were washed and then incubated for an additional hour with the anti-mouse IgG (1:1,000) peroxidase conjugate (Sigma, Germany). The reaction was developed by oxidation with o-dianisidine.

2.3.16. Construction of vectors containing the PreS1-GST protein coding region

PreS1 gene sequence (coding for aa 1-149) was generated by PCR using the gene sequence of WHsAg (NCBI accession no. M19183) as template and a preS1 specific primer pair PreS1 pDest (see table 7). The PCR conditions are summarized in table 8. Differing from table 8, the PCR annealing temperature for generation of WHV preS1 was 58 °C. The PCR product PreS1 was cloned into the pENTR™/SD/D-TOPO® vector. The correct reading frame of the insert was verified by sequencing. The ligation reaction, the propagation and the purification of the plasmid (Invitrogen, Germany) were performed according to the manufacturer's instructions as well as the recombination reaction between the pENTR™/SD/D-TOPO® clone containing the PreS1 gene and the pDEST®24 vector. The ligation reaction, the propagation and the purification of the plasmid were also performed according to the manufacturer's instructions. The E. coli DH5α strain was used for transformation of the plasmid. The cloning and protein-expression procedure was shown in a flow diagram (Figure 8).

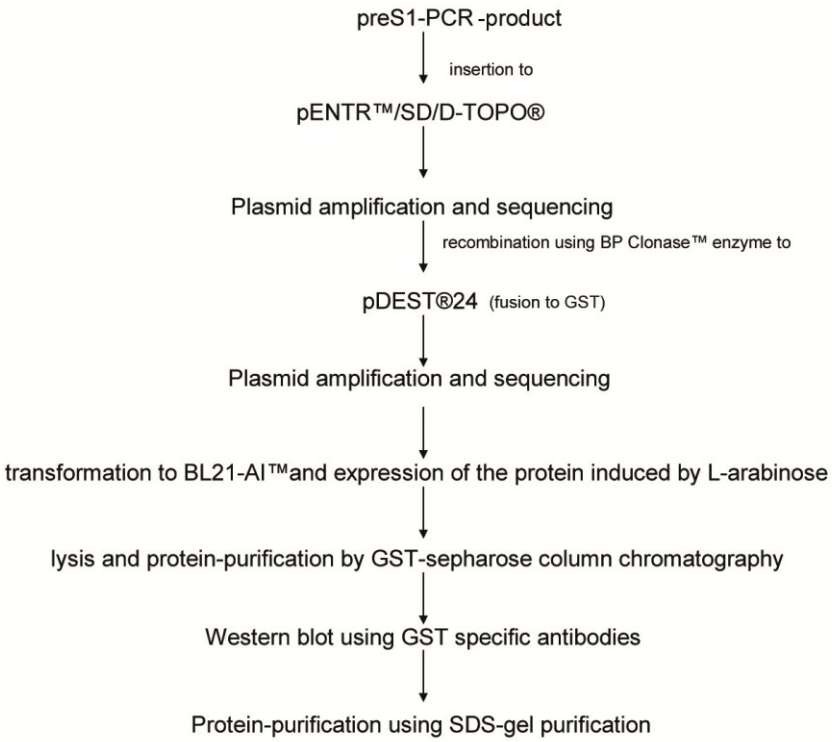

Figure 8: Flow diagram: Production of preS1-GST protein
The correct reading frame of the insert was verified by sequencing. Transformation of the plasmid into the BL21-AI™ strain (Invitrogen, Germany) allowed the expression of the corresponding protein induced by L-arabinose.

2.3.17. Purification of the PreS1-GST protein

After expression the PreS1-GST protein was purified by Glutathione Sepharose™ 4B Media/GSTrap columns according to the manufacturer's instructions (GE Healthcare Life Science, Germany). The purified protein was detected by SDS-gel electrophoresis and by Western blot as described above using an anti-GST antibody from goat (Amersham Biosciences, Germany). The second monoclonal antibody (anti-goat/sheep antibody peroxidase conjugated, Sigma, Germany) was used at a dilution of 1:5,000.

2.3.18. Production and purification of PreS1-GST protein using electro-eluter

The PreS1-GST specific band was cut out and was eluted from gel using electro-eluter. The membrane caps were soaked in protein elution buffer for at least 1 hour at 60°C.

Elution buffer: Tris-HCl:3 g (25 mM); Glycine: 14.4 g (192 mM);
SDS: 1 g (0.1%); Add water to 1 l

Thereafter the membranes were handled with gloves. The gel slice was loaded into the Model 422 Electro-Eluter. The protein was eluted at 8 mA/glass for 4 hours. The sample was stored at -20°C.

2.3.19. Electron microscopy

Virus like particles (VLPs) were visualized by electron microscopy in the Biomedical Center of Riga (Latvia) in the Institute of Prof. Pumpens as described before (Skrastina et al., 2008).

2.3.20. Statistical analysis

The Mann-Whitney test was used to compare humoral and cellular HBV-specific immune responses between the Sci-B-Vac and HBVAXPRO® group in healthy volunteers and WHV-PreS1 specific humoral immune responses in mice. P values of < 0.05 were considered as statistically significant.

3. Results

3.1. Immunization of healthy volunteers with HBV vaccine

Healthy volunteers, 30 persons in total, were immunized four times with Sci-B-Vac (n = 15) or HBVAXPRO® (control group, n = 15) at two-week intervals to determine B and T-cell immune responses to demonstrate whether Sci-B-Vac is suitable for short time immunization of living liver donors. Cellular immune responses (IFN-γ production and lymphocyte proliferation) were measured after stimulation of PBMCs with HBV surface proteins in weeks 0, 2, 4, 6, and 10. One year after immunization cellular immune responses were measured in three individuals vaccinated with Sci-B-Vac or HBVAXPRO®, respectively. Side effects were not observed in any of the vaccinated volunteers.

3.1.1. B-cell response in healthy volunteers

We examined the humoral immune response (anti-HBs titer) prior to each and 4 weeks after the last immunization (Fig. 9). Anti-HBs was undetectable before the first immunization (week 0). In nine of fifteen volunteers immunized with Sci-B-Vac anti-HBs was detected already after the first immunization (week 2: geometric mean value [GMT] 16 IU/l). In contrast, only one volunteer of the control group showed a humoral immune response at this time point. After the second immunization (week 4) the GMT of the anti-HBs titer increased in both groups (Sci-B-Vac group 151 IU/l; control group 3 IU/l) compared to the mean values measured after the first immunization. At this time point all except for one volunteer of the Sci-B-Vac group showed a humoral immune response to HBV, whereas only five of fifteen controls became anti-HBs positive. After the third immunization antibody production was detected in all volunteers immunized with Sci-B-Vac with a GMT of 226 IU/l. In the control group 5/15 probands still presented without antibody production (GMT of anti-HBs titer 12 IU/l). After the last immunization (week 10) all volunteers showed a humoral immune response (GMT in the Sci-B-Vac group 842 and 205 IU/l in the control group). One year after the first immunization we measured humoral immune responses in three volunteers immunized with Sci-B-Vac or HBVAXPRO®, respectively. The Sci-B-Vac vaccinated probands showed higher immune responses compared to those immunized with HBVAXPRO® (GMT of anti-HBs 5,318 IU/l in the Sci-B-Vac group and of 95 IU/l in the control group). In summary, the short immunization protocol Sci-B-Vac induces significantly higher antibody titers after each of the four vaccinations as compared to volunteers immunized with HBVAXPRO®.

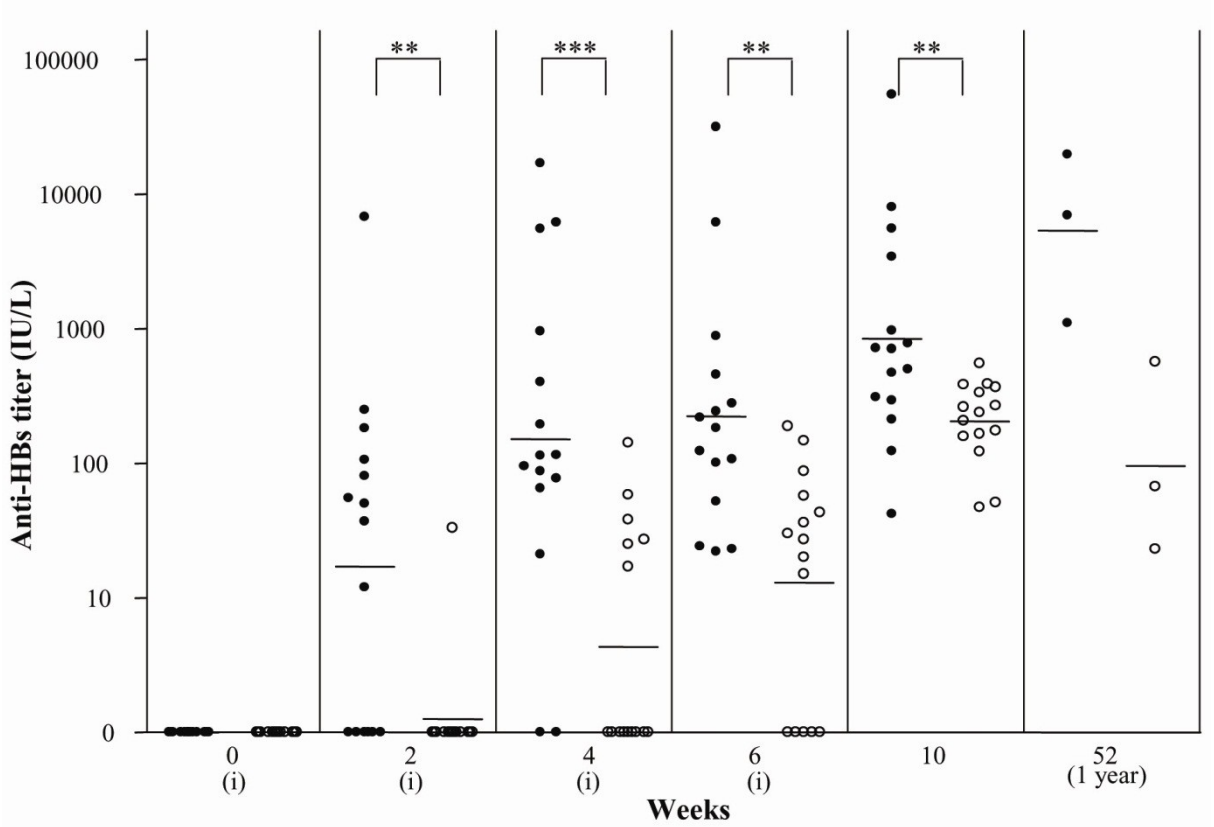

Figure 9: Humoral immune response after immunization with hepatitis B vaccines Sci-B-Vac (n = 15) and HBVAXPRO® (n = 15).
The X-axis indicates time points of analysis and of immunizations (i). The Y-axis shows anti-HBs titers in IU/l. The horizontal lines indicate geometric mean values. Volunteers immunized with [•] Sci-B-Vac or [o] the control vaccine. **P < 0.01 and *** P < 0.001.

3.1.2. T-cell response (Lymphocyte proliferation) in healthy volunteers

To determine cellular immune response to HBVAXPRO® and Sci-B-Vac in the short time immunization protocol cells of the volunteers in both vaccinated groups were stimulated with S protein, with PreS1 region of the L protein or with simultaneous stimulation with S, M and L protein.

After stimulation with S, M and L proteins both vaccinated groups did not display a proliferative response at week 2 (Fig. 10a). After the second immunization (week 4) cell proliferation became detectable in individuals immunized with Sci-B-Vac (mean value of SI = 9.5 vs. SI = 1.6 in the control group). The immune response increased after the third and fourth immunization (weeks 6 and 10) in both groups. It was significantly higher (P < 0.01) in volunteers immunized with Sci-B-Vac (mean values of SI = 17.7 and 26.5) than with the control vaccine (mean values of SI = 4.9 and 5.3). One year after immunization proliferative immune responses were still higher in Sci-B-Vac vaccinated individuals (SI = 15.5, 56.7, or 108.2, respectively) compared to the control group (SI = 17.2, 6.7, or 2.4). Thus, individuals

immunized with Sci-B-Vac showed significantly higher proliferative responses to S, M and L proteins compared to volunteers immunized with the control vaccine.

The PreS1 fragment of the L protein induced cell proliferation (Fig. 10b) in volunteers immunized with Sci-B-Vac after the last immunization (mean value of SI = 3.9 vs. SI = 0.9 in the control group). One volunteer immunized with HBVAXPRO® was, unexpectedly, measured positive after the third immunization but he remained negative after the fourth immunization. As expected, no proliferative response was measured in the remaining volunteers of the control group. One year after immunization a proliferative immune response was still detectable in one of three Sci-B-Vac vaccinated probands (SI = 11.9).

The cellular immune response was also measured after stimulation with the S protein (Fig. 10c). After the first and second immunization with Sci-B-Vac or the control vaccine (weeks 2 and 4) cell proliferation was found only in single volunteers. After the third immunization (week 6) proliferative responses were detected in a subgroup of volunteers immunized with Sci-B-Vac (mean value of SI = 2.0) and the control vaccine (mean value of SI = 4.2) and increased in the Sci-B-Vac group (mean value of SI = 2.7) after the last immunization (week 10). In contrast, cell proliferation slightly decreased in the control group at this time point (mean value of SI = 3.1). Thus, both groups showed similar cell proliferation after stimulation with the S protein alone. Of note, one year after immunization proliferative immune responses were still detected in three Sci-B-Vac (SI = 23.4, 7.5, or 3.2) and in two HBVAXPRO® (SI = 13.6 or 3.6) vaccinated probands.

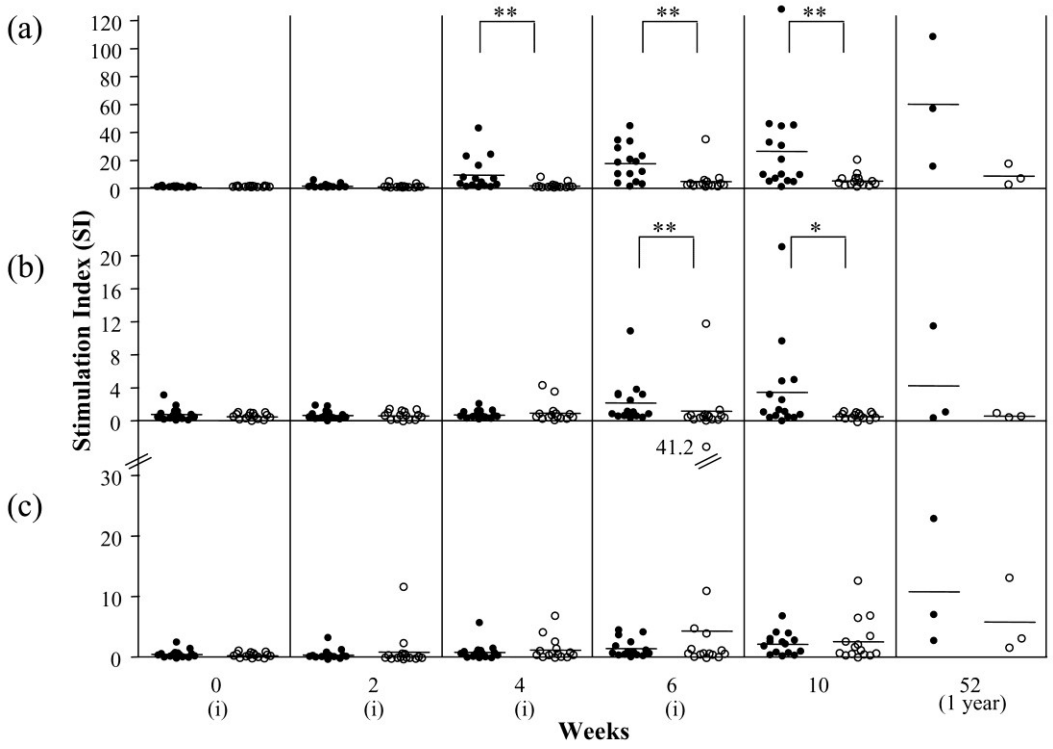

Figure 10: Cellular immune response (proliferation assay) after immunization with hepatitis B vaccines Sci-B-Vac and HBVAXPRO®.
The X-axis indicates time points of analysis and of immunizations (i). The Y-axis shows the ratio between stimulated and unstimulated cells [stimulation index (SI)] after incubation with (a) L, M, and S proteins; (b) L protein fragment (108 aa); (c) S protein. The horizontal lines indicate mean values. Volunteers immunized with [●] Sci-B-Vac or [○] the control vaccine. *P < 0.05 and ** P < 0.01.

3.1.3. T-cell response (IFN-γ production) in healthy volunteers

In addition to lymphocyte proliferation we also observed IFN-γ production in both vaccinated groups by ELISpot assay. IFN-γ production was detected after stimulation with S protein or with PreS1 fragment of L protein or after simultaneous stimulation with S, M and L proteins.

After simultaneous stimulation with S, M and L proteins (Fig. 11a) we detected IFN-γ production in 3 volunteers immunized with Sci-B-Vac (mean value: 6.7 spots versus 0.9 spots in the control group) already after the first vaccination (week 2). After the second immunization (week 4) IFN-γ production increased in this group (mean value 28.8 spots) and was detectable in the control group (mean value 8.5 spots) for the first time. Finally, volunteers immunized with Sci-B-Vac presented mean values of 57.1 and 71 spots after the third and fourth immunization (weeks 6 and 10). At these time points 20.1 and 42.2 spots were detected in the control group. Thus, Sci-B-Vac vaccinated volunteers showed earlier and significantly higher (P < 0.05) cellular immune responses after stimulation than controls did.

One year after immunization IFN-γ production in T-cells was still higher in the Sci-B-Vac vaccinated volunteers (67, 74, or 77 spots) compared to the control group (36, 40, or 56 spots).

The PBMCs were also stimulated with the PreS1 fragment of the L protein (108 aa). After the third and fourth immunization IFN-γ production was detected (Fig. 11b) at weeks 6 and 10 in the Sci-B-Vac group. In this group, mean values of 4.7 and 6.3 spots, respectively, were reached, whereas no response was measured in the control group as expected. One year after immunization IFN-γ production was still detectable in two of three tested Sci-B-Vac vaccinated volunteers (11 and 19 spots) whereas no response was measured in the control group. Taken together, only Sci-B-Vac vaccinated volunteers showed cellular immune responses to the PreS1 fragment of the L protein.

Additionally, the S protein was used for stimulation of PBMCs (Fig. 11c). After the first and second immunization (week 2 and 4) IFN-γ production in T-cells was detected in a single volunteer immunized with Sci-B-Vac (28 spots and 43 spots). After the third and fourth immunization (weeks 6 and 10) IFN-γ was detectable in the Sci-B-Vac group with mean values of 10.6 and 18.8 spots and in the control group with mean values of 6.9 and 22.7 spots, respectively. One year after immunization immune responses were still detectable in two volunteers immunized with Sci-B-Vac (27 or 28 spots) and in one volunteer immunized with HBVAXPRO® (60 spots). Thus, both vaccines induced similar cellular immune responses after stimulation with the S protein, which occurred earlier in one individual immunized with Sci-B-Vac.

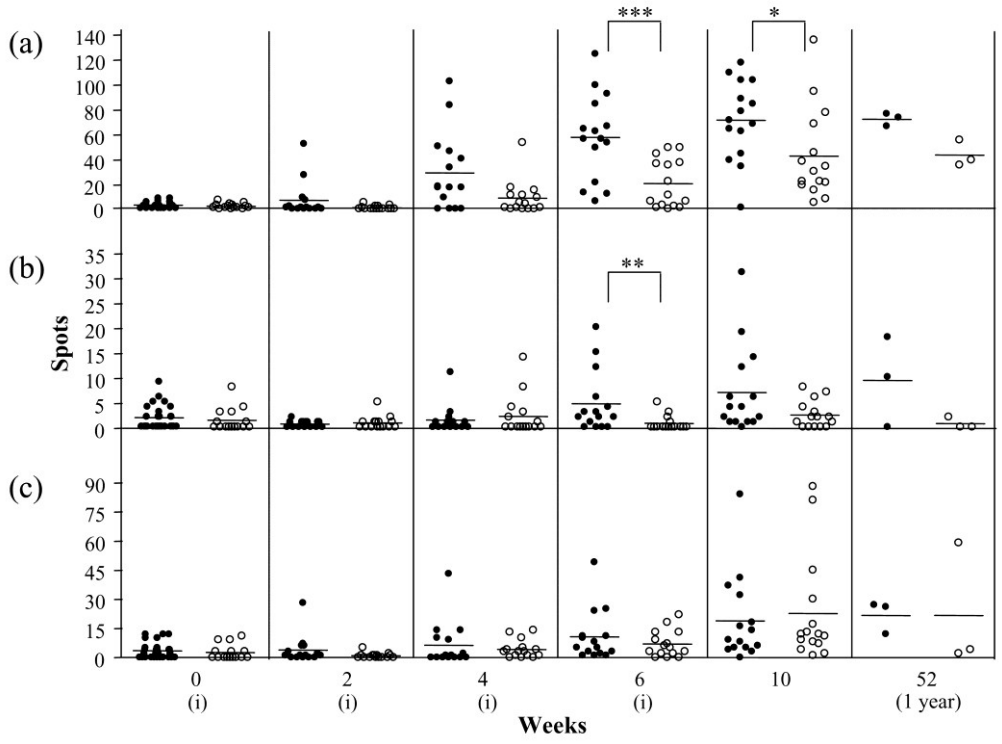

Figure 11: IFN-γ production (ELISpot) after immunization with the hepatitis B vaccines Sci-B-Vac (n =15) and HBVAXPRO® (n = 15).
The X-axis indicates time points of analysis and of immunizations (i). The Y-axis shows IFN-γ production (spots) after stimulation with (a) L, M, and S protein; (b) L-protein fragment (108 aa); (c) S protein. The horizontal lines indicate mean values. Volunteers immunized with [●] Sci-B-Vac or [○] the control vaccine. *P < 0.05, **P < 0.01, and *** P < 0.001.

3.2. Immunization of potential living liver donors

The aim was to show that adoptive immune transfer with the liver is feasible and can probably protect chronically HBV infected liver recipients from reinfection with the virus. In total 46 potential LLD were immunized with HBV vaccines (Sci-B-Vac or HBVAXPRO®) to induce HBV specific immune responses following the short time immunization protocol described before. Seven donors received one booster immunization with HBVAXPRO® because of preexisting vaccine derived HBV immunity. Humoral and cellular immune responses were measured in all 46 potential donors after the last immunization (Fig. 12). The number of immunizations differed from 1 to 5 due to the time available before transplantation or the time up to exclusion from donation. One donor received three booster immunizations using Sci-B-Vac prior to donation because no anti-HBs antibodies were detectable despite of two earlier immunizations.

3.2.1. B-cell response in potential living liver donors

The humoral immune response was measured in all potential donors 2-3 weeks after immunization (Fig. 12a). In the group of LLD who received only one immunization with Sci-B-Vac (n = 11) four showed detectable anti-HBs antibodies of up to 189 IU/l. In LLD immunized twice anti-HBs antibodies were detected in four out of seven donors with up to 825 IU/l. After three immunizations anti-HBs antibodies were present in all LLD (n = 11) with a peak of 2,069 IU/l. Seven of the fourfold immunized donors (n = 8) displayed anti-HBs titers between 44 and 9,831 IU/l. A single donor, who received five immunizations, showed an anti-HBs titer of 6,275 IU/l. A single booster immunization with HBVAXPRO® (n = 7) resulted in higher anti-HBs titers of up to 61,265 IU/l. The Sci-B-Vac boosted donor (3 immunizations) displayed an anti-HBs titer of 22,203 IU/l. As expected, the strength of humoral immune responses increased with the number of vaccinations. Groups of donors who received booster immunizations showed higher immune responses compared to those groups without previous immunizations.

3.2.2. T-cell response in potential liver donors

Cellular immune responses were detected using proliferation assay and IFN-γ ELISpot (Fig. 12b and 12c). In donors (n = 11), who received one immunization with Sci-B-Vac, three displayed cellular immune responses in the proliferation assay (SI of up to 11.9) and one of them in the ELISpot (70 spots). Donors, who were immunized twice (n = 7), showed comparable results. Two of them showed lymphoproliferative responses (SI of up to 44.6) and one displayed 12 spots in the ELISpot. After three immunizations the cellular immune response increased. Ten out of eleven donors who received three immunizations showed cellular immune responses in the proliferation assay (SI of up to 22.4) and seven of them in the ELISpot (up to 103 spots). In fourfold immunized donors (n = 8) cellular immune responses were detected in five donors in the proliferation assay (SI of up to 32.5) and four donors displayed up to 34 spots in the ELISpot. A single donor who received five immunizations displayed the highest cellular immune responses. An SI of 110.2 was detected in the proliferation assay and 91 spots were obtained in the ELISpot. However, donors (n = 7) who received one booster immunization with HBVAXPRO® displayed also substantial SI values of up to 23.4 in the proliferation assay and 75 spots in the ELISpot. The threefold booster vaccinated (Sci-B-Vac) donor showed lower cellular immune responses with an SI of 3.3 and 22 spots. In summary, the magnitude of cellular immune responses also correlated with the number of administered vaccinations.

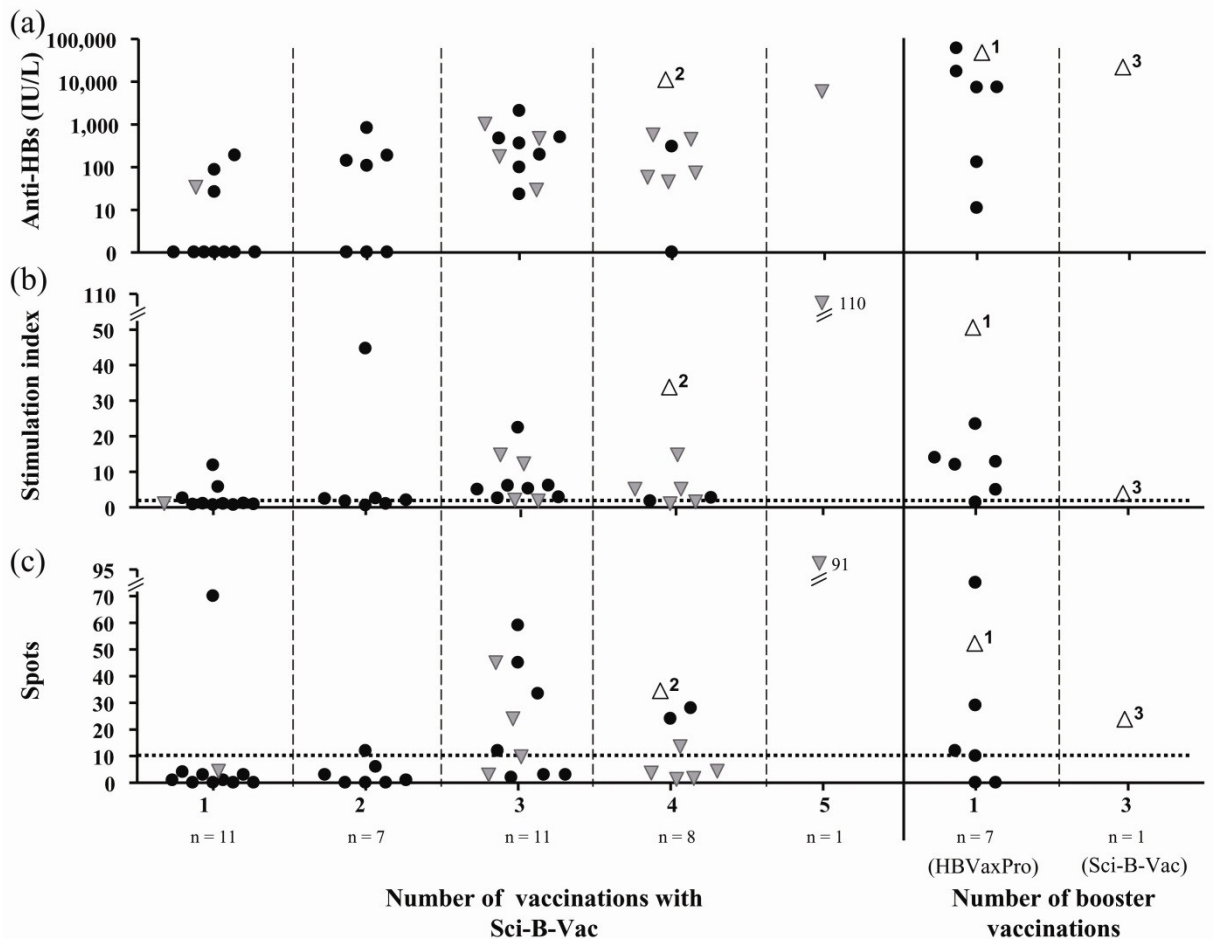

Figure 12: Humoral and cellular immune responses of 46 potential LLD after last immunization.
The x-axis indicates the number of vaccinations or booster-vaccinations in 46 potential living liver donors.
32 donors (●) were excluded from transplantation due to medical or psychological reasons. 14 donors (▼Δ) donated a part of their liver. Values in donors who transferred their HBV specific immunity are depicted as hollow triangles (Δ) with numbers 1-3. a) Anti-HBs titers in the LLD prior to transplantation or after the last immunization, b) cellular immune response in the proliferation assay, and c) in the IFN-γ ELISpot. The horizontal line indicates the cut-off (stimulation index of 2.5 or 10 spots, respectively).

3.2.3. Immune response in LLD donating the liver

In LLD humoral and cellular immune responses were measured before transplantation. However, only fourteen of the 46 potential LLD donated a part of their liver, while 32 were excluded from transplantation due to medical or psychological reasons. Three of the fourteen donors who donated the liver to a recipient showed anti-HBs titers > 9,000 IU/l (Fig. 12a) before transplantation. These three donors transferred their HBV specific immune responses to the recipients, while donors showing lower anti-HBs titers (n=11) did not. These three donors with high antibody titer after vaccination showed also cellular immune responses (Fig.

12b, c). Prior to the transplantation SIs of 3.3, 32.5 and 50.0 were detected in the proliferation assay and 52, 34 and 22 spots in the ELISpot, respectively.

3.3. Characteristic of adoptive transfer of immunity to HBV

Only in three of fourteen recipients the adoptive transfer was observed. In these three donor/recipient pairs presenting with an adoptive immune transfer the course is described below in detail.

3.3.1. Description of immune transfer in donor/recipient pair 1

In January 2006 a male donor (25 years) was evaluated for living liver donation for his HBV negative mother (55 years). She received the liver due to cryptogenic liver cirrhosis. The donor had already been immunized against HBV and showed an anti-HBs titer of 10,413 IU/l. After one booster immunization with HBVAXPRO® an increase of the anti-HBs titer to 57,500 IU/l was detected in the donor prior to transplantation (Fig. 13a).

During the surgical treatment the patient received plasma products. Two weeks after transplantation an anti-HBs titer of 1,800 IU/l was detected. This could partly be due to low amounts of antibodies in the blood products. To demonstrate that antibodies were transferred with the liver and produced by B-cells of the donor we calculated the amount of anti-HBs which was passively transferred with blood products. During the surgical treatment she received a total anti-HBs amount of 380 IU/l. The decrease of these antibodies based on the average half-life of 21 days is calculated and visualized in Figure 13a. The amount of antibodies measured in the recipient, however, clearly exceeds this value and therefore, can be attributed to anti-HBs antibodies transferred to or produced by B-cells of the recipient. An anti-HBs titer of 100 IU/l in the recipient one year after transplantation suggests that anti-HBs was actively produced in this patient since passively transferred anti-HBs antibodies are unable to persist over such a long period of time. A booster immunization with HBVAXPRO® at month 12 after transplantation induced, however, no increase of the antibody titer so far.

In addition, the recipient showed cell proliferation after stimulation with the L-HBsAg (SI of 4.9) at month 6 after transplantation, but at month 12 proliferation was no longer detectable. A booster immunization at this time point induced again an HBsAg specific cell proliferation

(SI of 3.1, month 13) (Fig. 13b). ELISpot results were negative in the recipient after transplantation (data not shown).

To summarize, in this HBV naïve recipient, who received a part of the liver from an immunized donor, we detected the transfer of HBV specific humoral and in addition of cellular immune responses.

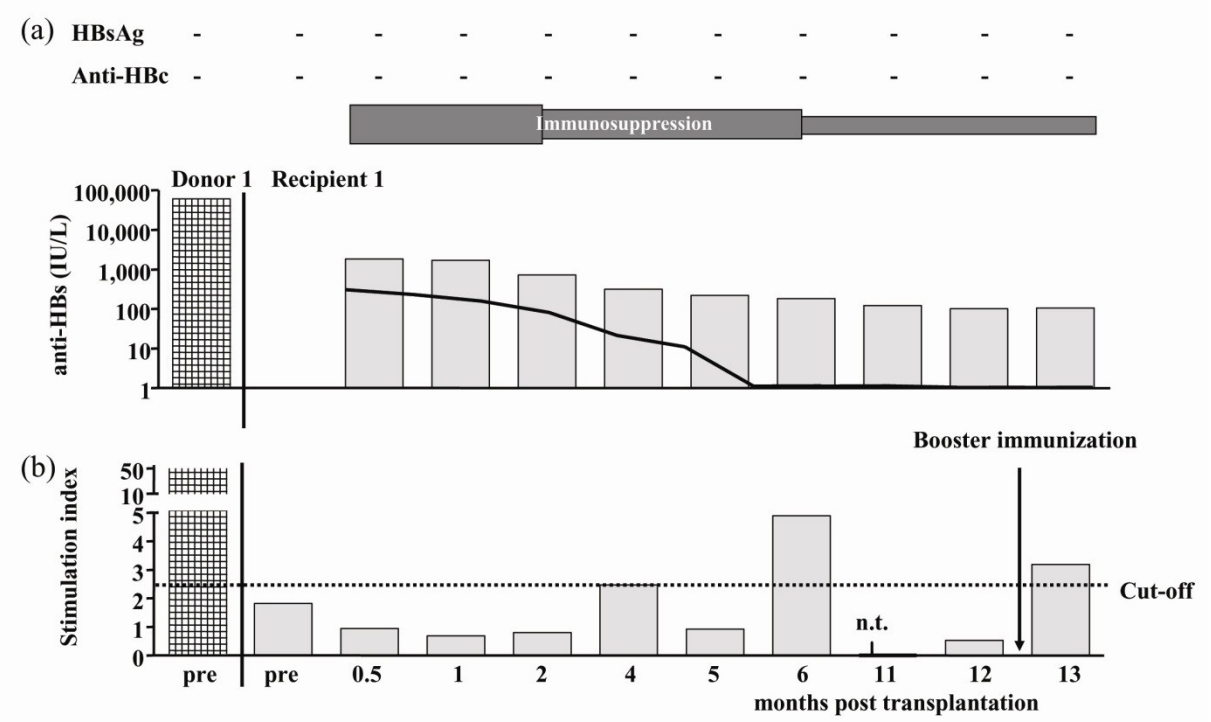

Figure 13: Donor/recipient pair 1: HBV specific immunity of the donor prior to transplantation and the recipient pre and post transplantation.
The X-axis indicates time points of analysis. a) The Y-axis shows anti-HBs titers in IU/l of the donor (▦) and the HBV negative recipient (▭). The curve indicates the half value time of anti-HBs derived from blood products during the transplantation. Negative (-) values for HBsAg and anti-HBc detection are indicated at the top. The size of the horizontal bars on the top indicates the amount of immunosuppressive therapy. b) The Y-axis shows the ratio between stimulated and unstimulated proliferation (stimulation index) of the donor and the HBV negative recipient using L-HBsAg as stimulus. The horizontal line indicates the cut-off (stimulation index of 2.5). n.t.: not tested.

3.3.2. Description of immune transfer in donor/recipient pair 2

In September 2006 a female HBV negative LLD (48 years old) was evaluated for living liver donation for her HBV negative sister (49 years old). She received the graft due to multiple liver abscesses. The donor was immunized against HBV in a short time immunization protocol using Sci-B-Vac vaccine (4 injections in 2 weeks intervals) and an anti-HBs titer of 9,831 IU/l was observed prior to transplantation (Fig. 14a).

The anti-HBs titer of the recipient measured at week 2 (10 IU/l) after transplantation was likely due to low amounts of antibodies in blood products given during the transplantation. Subsequent samples of this patient were anti-HBs negative (Fig. 14a). Already one month after transplantation, however, the recipient showed lymphoproliferation after stimulation with the L-HBsAg (SI of 7.1) (Fig 14b). Again, positive results were observed at months 5 (SI of 3.1) and 6 (SI of 40) after transplantation. Furthermore, a transfer of cellular immune response was also demonstrated by IFN-γ ELISpot at month 6 (22 spots) (Fig 14c).

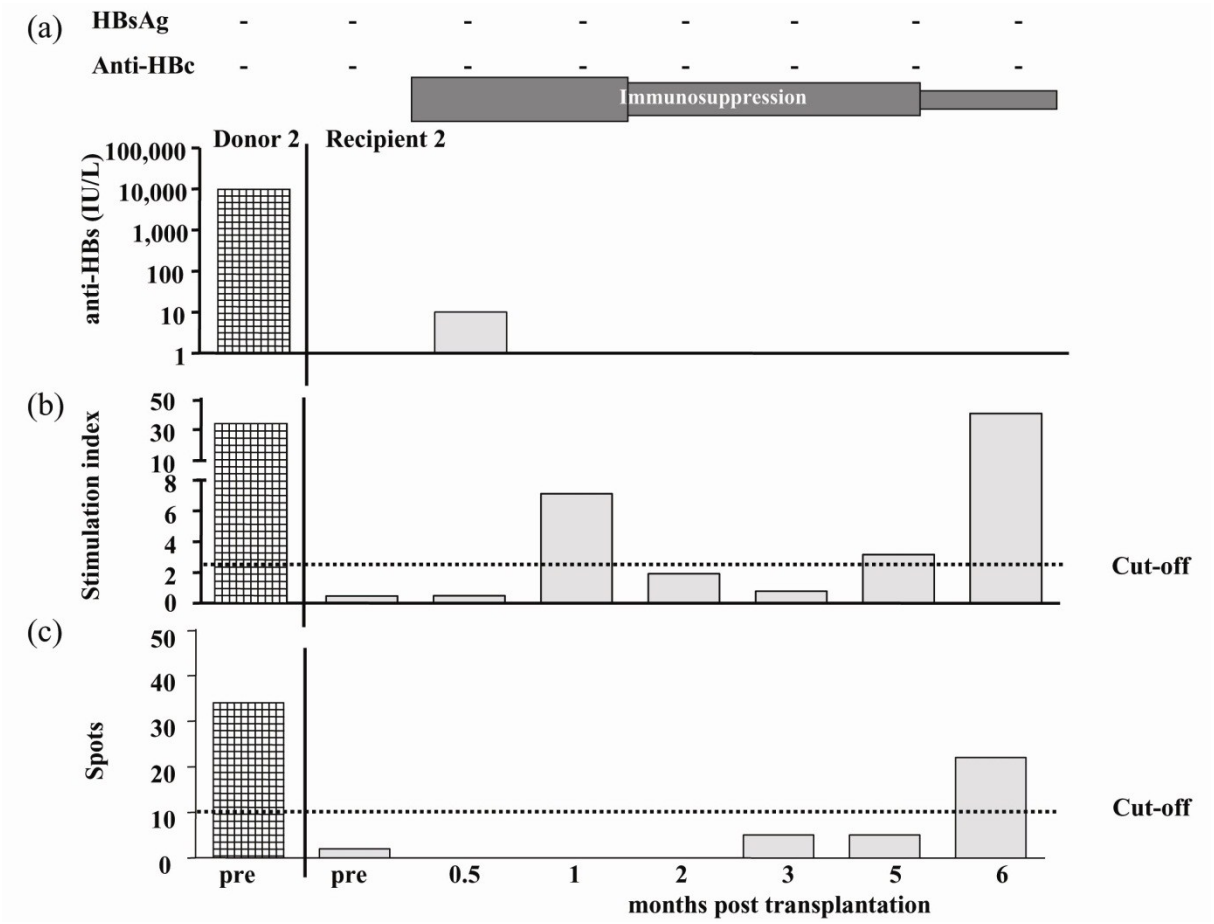

Figure 14: Donor/recipient pair 2: HBV specific immunity of the donor prior to transplantation and the recipient pre and post transplantation.
The X-axis indicates time points of analysis. a) The Y-axis shows anti-HBs titers in IU/l of the donor (▦) and the HBV negative recipient (▭). Negative (-) values for HBsAg and anti-HBc are indicated at the top. The size of the horizontal bars on the top indicates the amount of the immunosuppressive therapy. b) The Y-axis shows the ratio between stimulated and unstimulated proliferation (stimulation index) of the donor and the HBV negative recipient using L-HBsAg as stimulus. The horizontal line indicates the cutoff. c) The Y-axis shows the IFN-γ production (spots) measured by ELISpot in donor and recipient after incubation with the L-HBsAg. The horizontal line indicates the cut-off (10 spots).

Despite of a high anti-HBs titer in the donor, only cellular immune responses were measurable in the recipient due to the transfer of HBV specific T-cells with the liver. The absence of a humoral HBV specific immune response in the recipient could be explained by a medication with rituximab prior to transplantation and several plasmapheresis. This therapy was necessary to overcome blood group incompatibility of the donor and the recipient. Several examinations by flow cytometry have shown that no or extremely low numbers of B-cells persisted in this recipient (data not shown).

3.3.3. Description of immune transfer in donor/recipient pair 3

In January 2006 a female HBV negative LLD (30 years) was evaluated for living related liver donation for her chronically HBV infected father (58 years). He was transplanted because of HBV infection related liver cirrhosis and hepatocellular carcinoma. The donor did not show a measurable anti-HBs titer at the time of enrolment despite previous documented vaccinations in September 2000 and January 2001 with 10 µg of HBVAXPRO®. This donor was immunized three times with 20 µg of Sci-B-Vac at monthly intervals. Prior to transplantation an anti-HBs titer of 22,200 IU/l was detected (Fig. 15a).

During transplantation the recipient received blood products and HBIg (Hepatect, 286 IU/l daily) was administered after transplantation for a period of 21 days to prevent reinfection of the new liver (Fig. 15a). The anti-HBs antibodies measured on day 6 after transplantation (120 IU/l) could on the one hand be due to low amounts of antibodies in blood products given during the transplantation, or on the other hand to HBIg administration. To demonstrate that anti-HBs antibodies were transferred with the liver and produced by B-cells of the donor or/and the patient we calculated the amount of HBIg derived antibodies. According to this, the antibodies which were passively transferred achieved the highest concentration at day 21 with 4,681 IU/l. At day 14 after transplantation, however, a substantial antibody titer of 43,040 IU/l was detected in the recipient. At day 21 and at week 5 after transplantation the anti-HBs titer increased to 47,000 and 57,993 IU/l, respectively, and decreased at weeks 8 (anti-HBs of 49,965 IU/l), 14 (anti-HBs of 8,397 IU/l), 19 (anti-HBs of 226 IU/l), and 26 (no anti-HBs detected). Therefore, the anti-HBs titer peak of 57,993 IU/l at week 5 after transplantation in the recipient cannot be attributed to HBIg medication. A transfer of the humoral immune response with the liver graft is assumed.

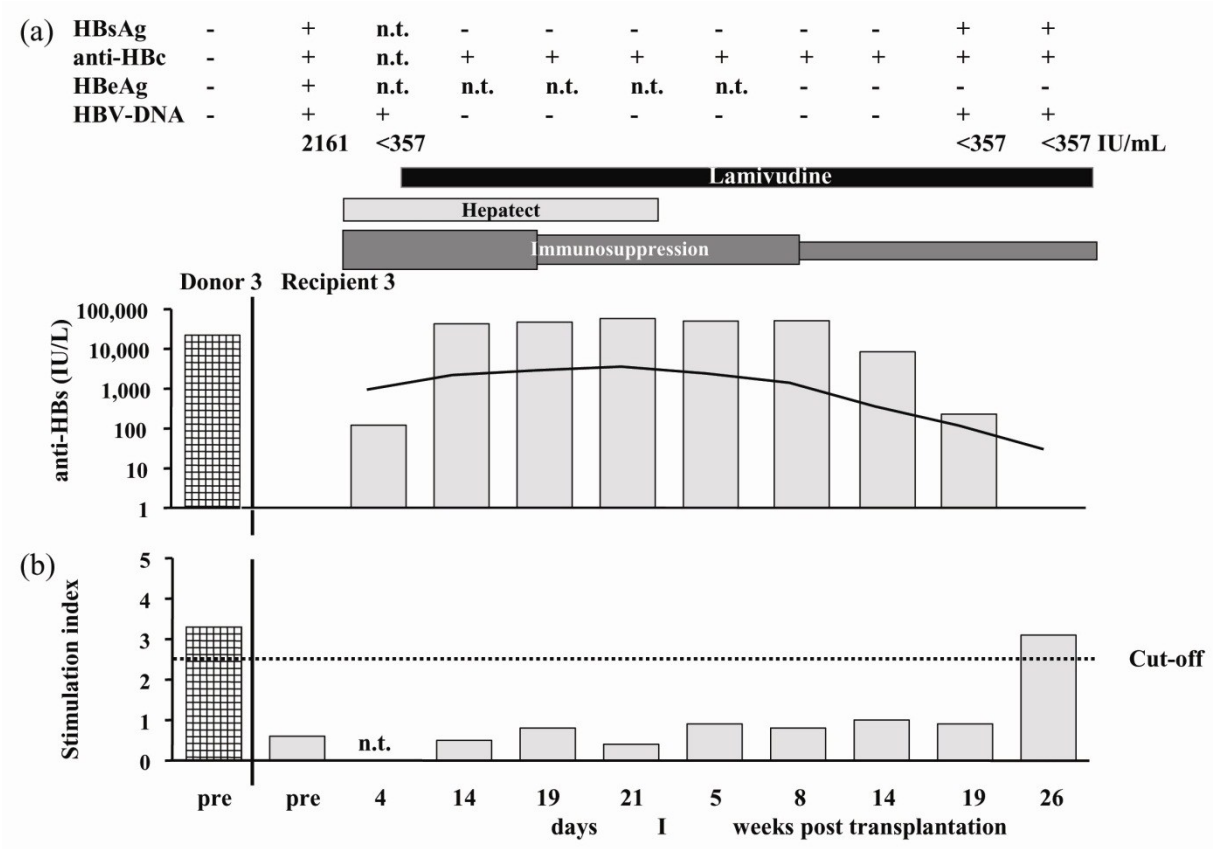

Figure 15: Donor/recipient pair 3: HBV specific immunity of the donor prior to transplantation and the chronically HBV infected recipient pre and post transplantation. The X-axis indicates time points of analysis. a) The Y-axis shows anti-HBs titers in IU/l of the donor (▦) and the chronically HBV infected recipient (▭). Antiviral therapy with lamivudine is visualized at the top with a horizontal black bar. The curve shows the level of anti-HBs due to HBIg medication (286 IU/l daily) considering the average half-life of the antibodies. Positive (+) or negative (-) results of HBsAg, anti-HBc, HBeAg, and HBV specific qualitative and quantitative PCR are indicated at the top. b) The Y-axis shows the ratio between stimulated and unstimulated proliferation (stimulation index) of the donor and the chronically HBV infected recipient after incubation with the L-HBsAg. The horizontal line indicates the cut-off (stimulation index of 2.5). n.t.: not tested.

To show the difference in the course of anti-HBs titers between patient 3 and a chronically HBV infected recipient, who received the liver from a donor without HBV immunity; we compiled the serological results of one exemplary patient (Fig. 16). Anti-HBs titers of up to 2,300 IU/l (week 26) could be observed in this chronically HBV infected recipient after transplantation. HBIg was administered to the recipient for 11 days with a daily dose of 286 IU/l after the surgical treatment. In addition the same Hepatect dose (286 IU/l daily) was administered for 10 days at weeks 8 and 9 as well as at weeks 19 and 20 after transplantation. The half life time of Hepatect derived antibodies was calculated and a peak of 2,988 IU/l (week 8) is visualized in Figure 15.

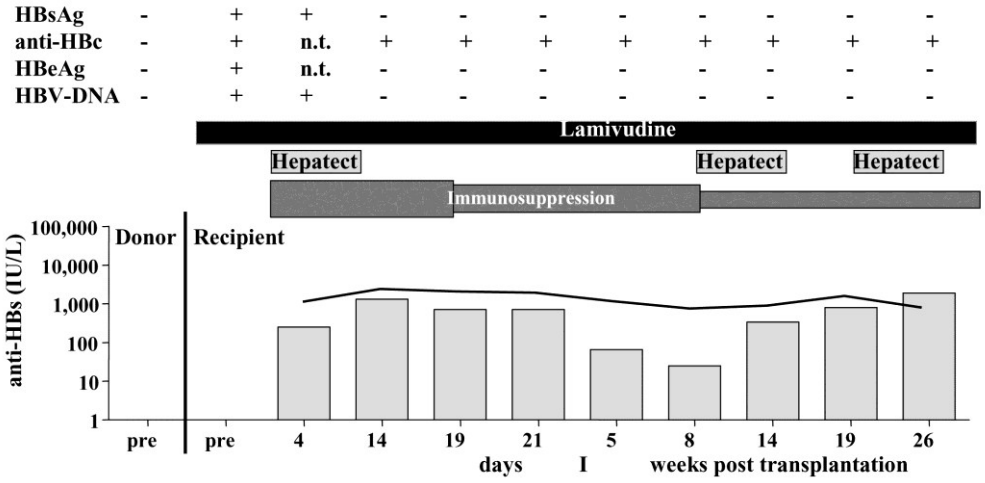

Figure 16: Humoral immune response of a chronically HBV infected recipient who received the liver from a donor without HBV immunity.
The X-axis indicates time points of analysis. Positive (+) or negative (-) results of HBsAg, anti-HBc, HBeAg, and HBV specific qualitative PCR are indicated at the top. The Y-axis shows anti-HBs titers in IU/l of the donor and the chronically HBV infected recipient (▭). The administration of HBIg (Hepatect) for 11 days after transplantation and for 10 days at week 8 and 9 as well as at week 19 and 20 (286 IU/l daily) are visualized as grey bars. Before and after transplantation the recipient received lamivudine therapy as indicated with a black bar. Further, the reduction of immunosuppressive therapy is shown with the size of the horizontal bar.

The amount of anti-HBs, which was measured in this chronically HBV infected patient can usually be observed in recipients due to HBIg medication (data not shown). The anti-HBs antibodies in recipient 3 were more than 1 log higher than in the control patient and, therefore, not solely due to HBIg administration.

To show that the vaccine which was used for the immunization of the donor is suitable to overcome the chronic infection in the recipient, we examined the HBV genotype and subtype persisting in the recipient. The vaccine, Sci-B-Vac, which was used for the immunization of the donor, is based on A2/HBsAg genotype and subtype adw2. The recipient was infected with an HBV isolate of the same genotype and subtype (A2/HBsAg adw2). Therefore, the vaccine was suitable to overcome the infection in the recipient. The specificity of anti-HBs antibodies in the recipient was tested. These antibodies were neutralized by all three different HBsAg subtypes (adw, adr, and ayw) indicating that the major portion of anti-HBs was directed to the a-determinant (data not shown). Thus, anti-HBs antibodies present in the patient after transplantation were likely to possess the ability to neutralize HBV viral

particles. A part of the circulating anti-HBs antibodies, however, derived from HBIg administration.

In addition to the humoral immune response to HBsAg, cellular immune responses in donor/recipient pair 3 were measured in proliferation assays. Prior to the transplantation an L-HBsAg protein specific immune response (SI of 3.3) was detected in the donor and no immune response in the recipient (Fig. 15b). At week 26 after transplantation the recipient developed an L-HBsAg protein specific cellular immune response with an SI of 3.1. No immune responses could be detected in the recipient before and after transplantation by ELISpot (data not shown).

We measured HBsAg, HBeAg and HBV DNA one week before and at several time points after the transplantation in patient 3. Prior to transplantation these three parameters were positive. At day 4 after transplantation a low level of HBV DNA was detected despite the presence of anti-HBs. Therefore, antiviral therapy with lamivudine was started on day 5 after transplantation. Afterwards, HBV DNA was no longer detectable. From week 19 onwards low levels of HBV DNA (< 357 IU/ml) and HBsAg were detected again in the serum. However, no HBeAg could be measured. Unfortunately, further observation was not possible, because the patient died at week 28 after transplantation due to perforating lung metastases.

3.3.4. Summary of the adoptive immune transfer

The follow up of three patients indicates that adoptive immune transfer with the liver is possible in humans. However, as shown in donor/recipient pair one and three, high titer of anti-HBs (> 9,000 IU/l) is required to transfer the humoral immune response from donor to the recipient. In addition, we were able to show that cellular immune response can also be transferred from donor to HBV naïve recipient (donor/recipient pair 1 and 2). In addition, transfer of HBV specific immunity to a chronically infected recipient is possible (donor/recipient pair 3). However, additional patient pairs have to be investigated to determine whether the adoptive immune transfer can protect chronically HBV infected patients from reinfection with the virus. In addition immunization of chronically infected patients after adoptive immune transfer should be performed to boost transferred HBV specific immune response.

3.4. Characterization of WHV virus like particles (VLP)

In the first two parts of the studies we increasingly investigated adoptive transfer of immunity by liver transplantation. These experiments showed that adoptive immune transfer is only possible after induction of high titers of HBV specific antibodies in donors. These experiments encouraged us to examine a new type of hepadnaviral vaccine to enhance the immune response to PreS1. So far the most HBV vaccines do not contain PreS1 region of the HBV L protein. In several studies a PreS1 peptide was demonstrated to interact with the receptor of hepatocytes. PreS1 peptide fused to the hepadnaviral core protein virus-like particles (VLPs) represents an interesting approach to improve hepatitis B vaccines. Thereby, a broader range of virus neutralizing protective antibodies could be induced resembling the repertoire of protective antibodies acquired during natural infection. Such vaccine inducing both the humoral and cellular immune response may qualify for prophylactic immunization as well as for therapeutic vaccination of chronic HBV carriers.

As a preclinical model we used woodchuck (*Marmota monax*) which can be infected with the woodchuck hepatitis virus (WHV). WHV is a member of the family Hepadnaviridae and is closely related to HBV (Lu et al., 2001). The generated VLPs are composed of WHV core proteins with insertion of WHV PreS1-derived peptides. In the future the WHV model will be used to proof if generated particles are suitable for therapeutic and/or prophylactic vaccination.

3.4.1. Prediction of the major immunodominant region (MIR) of WHV core.

The structure of HBV core particles was already published in 1994 (Crowther et al., 1994). One of the most interesting sites for insertion of epitopes into HBV core protein is the major immunodominant region (MIR). This region is located in the loop region aa 74-89. The MIR of WHV core protein was, unfortunately, not defined during the performance of this project but the sequence of WHV core protein was already known. Due to a high homology between HBV and WHV core protein of about 80 % we used the known structure of HBV core to predict the structure of WHV core. Therefore we used the 3D-JIGSAW software (see Methods section 2.3.12). This application is able to build three-dimensional maps based on homologies of highly resolved structures. Due to findings based on this technology we defined the spike of WHV core truncated protein near to the region between aa 74-78 (Fig. 17).

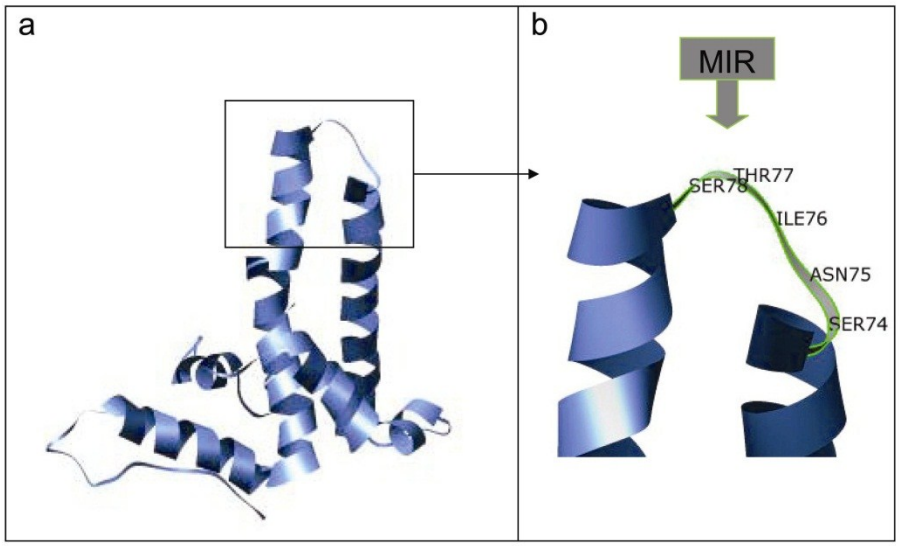

Figure 17: Predicted 3D structure of WHV core protein.
a) Structure of truncated WHV core protein (149 aa residues). b) Major immunodominant region (MIR) of truncated WHV core protein with amino acid sequence (3 letter code) and the position within the expected loop region (Bates et al., 2001).

3.4.2. Construction of plasmids containing WHcAg aa 1-149 with PreS1 Pep1-Pep6 inserts.

One aim of these experiments was to insert the PreS1-fragment, which induce neutralizing antibodies, into a WHV core VLP. For HBV the PreS1 region, which induces neutralizing antibodies, is known. Unfortunately, this region has not yet been defined for WHV PreS1. The homology of HBV PreS1 and WHV PreS1 protein- sequence is unfortunately very low; therefore we were not able to predict the region, which is necessary to binde to the receptor of hepatocytes. Therefore, we decided to create six VLP-constructs with partial sequences of WHV PreS1 starting from the n-terminus and in the sum bearing the whole WHV PreS1 fragment. Six overlapping PreS1 fragments (for protein- and DNA-sequence see appendix 7.2), corresponding to the whole PreS1 part of the L protein, were inserted into the major immunodominant region (MIR) of the WHcAg.

The PQE60 plasmid containing WHcAg region including Bsp119 restriction site in the MIR of WHcAg was kindly provided by Dr. Menji Lu. The PCR products of the six PreS1 peptides (Pep1-Pep6) were amplified using PCR primers including Bsp119 restriction sites. Plasmid and PCR-products were digested by Bsp119 enzyme and ligated using T4 DNA ligase (see Methods 2.3.13). All generated constructs were verified by DNA sequencing (for sequence see section 7.1 of the appendix). The schematic illustration of the constructs was shown in figure 18.

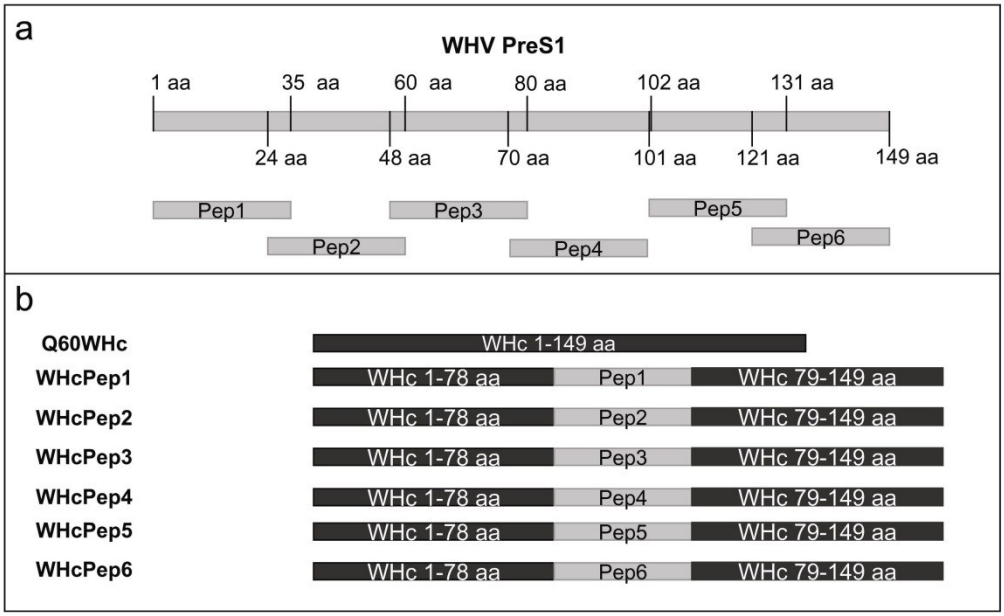

Figure 18: WHV PreS1 peptides and their position after insertion into the truncated WHV core protein.
a) Schematic illustration of WHV PreS1 protein and the position of PreS1 peptides (Pep1-Pep6). b) Schematic illustration of wild type core protein and WHV core constructs with Pep1-Pep6 insertions between aa 78 and aa 79. Used protein names are listed on the left.

3.4.3. Purification of capsids

Six constructs WHcPep1-WHcPep6 that encoded the truncated WHcAg with PreS1 fragments as inserts (Pep1-Pep6) were used to test their ability of protein expression, solubility, formation and stability of VLPs. The wild type truncated WHcAg construct Q60WHc has been used as a control. The constructs were transformed into E. coli strain BL21. After approximately 3 hours expression in growth medium at 37°C, bacteria were lysed and protein expression and solubility of the proteins were examined by PAGE-electrophoresis. The distribution of soluble and insoluble proteins were examined by Western blot using HBV antibody anti-HBc mAb 14E11 which is cross-reactive to WHV core and was kindly provided by Prof. Dr. Paul Pumpens (Fig. 19).

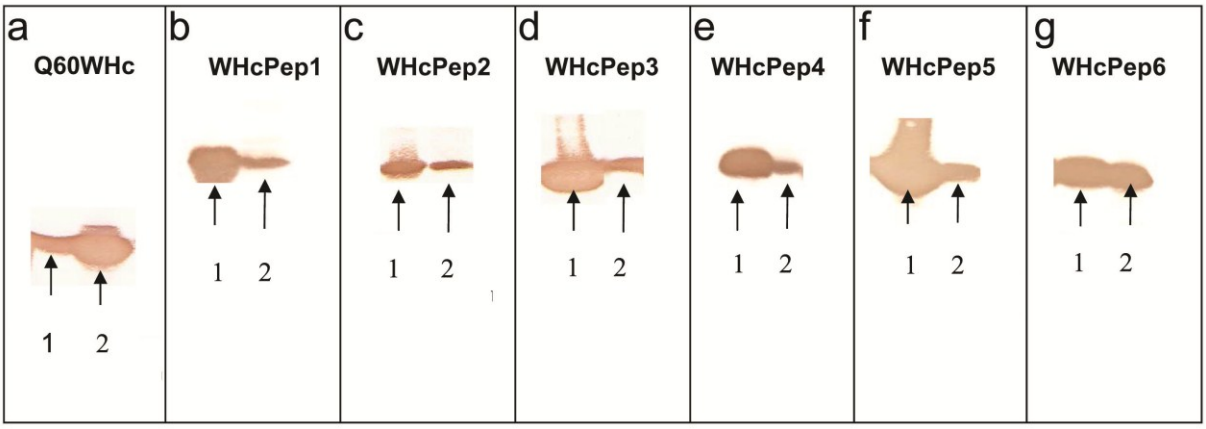

Figure 19: Examination of the solubility of expressed proteins.
The insoluble (1) and the soluble (2) part of the expressed proteins were detected by Western Blot. a) Q60WHc, b) WHcPep1, c) WHcPep2, d) WHcPep3, e) WHcPep4, f) WHcPep5 and g) WHcPep6

The wild type core protein (Q60WHc) was expressed and a large part of the protein was soluble as shown in the Western blot (Fig. 19a). The electromicroscopic investigation confirmed that Q60WHc formed VLPs (Fig. 20a).
The experiments with chimeric proteins (expressed from constructs: WHcPep1-WHcPep6) demonstrated different particle formation (Fig. 20b-g and table 9).

Table 9: Conditions, methods and results of VLP production

particle	expression	solubility	lysis	concentration	stability
WHc wildtype	high	high	lysozyme	28 mg/ml	high
WHcPep1	high	low	sonication	0.6 mg/ml	very low
WHcPep2	moderate	very low	sonication	incapable of measurement	n.d.
WHcPep3	high	low	sonication	2.6 mg/ml	moderate
WHcPep4	high	low	aluminium oxide	2.8 mg/ml	moderate
WHcPep5	high	low	sonication	incapable of measurement	n.d.
WHcPep6	high	moderate	sonication	0.6 mg/ml	low

Three methods, aluminium oxide, lysozyme or sonication, respectively, were used for lysis of the bacteria. For each construct we selected the lysis method, which provided the most efficient transfer of the chimeric protein into the soluble part. In some cases, 1.5 M urea was added to dissolve particles. Higher urea concentrations disrupted the formation of VLPs. All expressed proteins formed VLPs as demonstrated in electro microscopic figure 20.

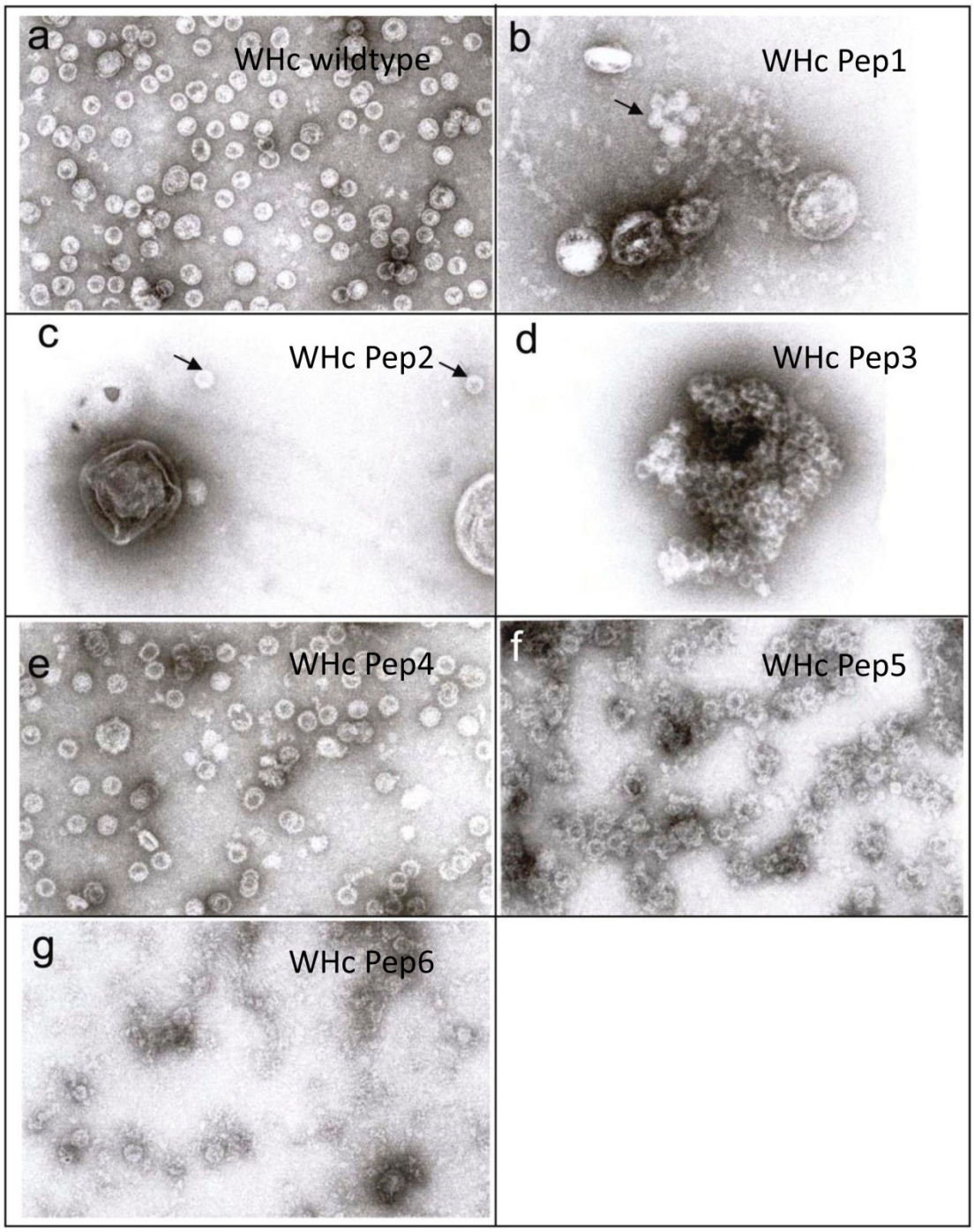

Figure 20: Electron microscopic pictures of the VLPs.
VLPs formed after purification of a) Q60WHc, b) WHcPep1 protein, d) WHcPep3 protein, e) WHcPep4 protein and g) WHcPep6 protein (in 50% glycerol). Particles are highlighted by an arrow. c) WHcPep2 and f) WHcPep5 were not suitable for purification. Particles were visualized after lysis.

After purification WHcPep1 degraded very fast and particles could not be used for immunization studies. Most VLPs expressed from construct WHcPep2 were insoluble and to low for purification. The stability after purification of VLPs expressed from WHcPep5 was very low; therefore no particles for immunization studies could be used. Nevertheless, VLPs expressed from three constructs (WHcPep3, WHcPep4 and WHcPep6) were purified and

could be used for immunization of mice. For the lysis we used sonication in case of WHcPep3 and WHcPep6. Aluminium oxide was used as lysis method for bacteria expressing WHcPep4 (see section Methods 2.3.14).

3.4.4. Production of the WHV PreS1-GST protein

To measure antibodies to WHV PreS1 we established an ELISA. Therefore the whole WHV PreS1 fragment fused to GST was generated to be used as an antigen. To generate PreS1 (aa 1-149)-GST protein, the preS1 gene was inserted into the pDest24 vector using GATEWAY technology as already described (see Methods 2.3.16 and Fig. 21).

After cloning, the preS1-gst containing section of the vector was checked by sequence analysis. The plasmid that encoded the estimated sequence (plasmid PreS1-GST) was used for transformation of E. coli strain BL21. After growing of the transformed E. coli and the induction of the protein expression, the bacteria cells were lysed and the expressed protein was purified by the GST-sepharose column chromatography (see Methods 2.3.17). The protein was visualized using GST specific antibodies in Western blot. The undigested protein (46 kDa) was purified again using SDS-gel purification, as degraded products were identified (Fig. 20a). The purified protein (Fig. 21b) with a concentration of 0.15 mg/ml was stored at − 20°C. For ELISA, wells were coated with a concentration of 150 ng PreS1-GST per well.

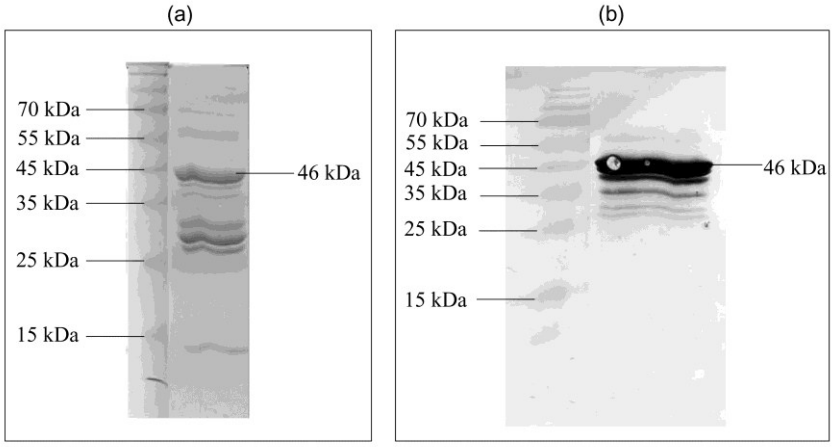

Figure 21: Purification of the PreS1-GST protein.
(a) SDS-gel electrophoresis after GST-sepharose column chromatography (coomassie staining). The left lane shows the protein ladder. The 46 kDa band on the right lane indicates the PreS1-GST protein. The remaining bands show degraded protein. (b) GST specific Western blot (using anti-GST antibody see 2.3.17) after SDS-gel purification. The 46 kDa band indicates the PreS1-GST protein.

3.4.5. Immunization of BALB/c mice

The aim of our experiments was to induce stronger and faster humoral immune response as compared to immunization with peptides. In subsequent experiments we immunized 16 BALB/c mice with VLP constructs WHcPep3 (aa 48-80) (n = 4), WHcPep4 (aa 70-101) (n = 4), WHcPep6 (aa 121-149) (n = 4) and with n-terminal PreS1 fragment aa 1-81 (n = 4). In two-week intervals we determined humoral immune responses to demonstrate that the purified VLPs are suitable to induce better immune response compared to immunization with the PreS1 fragment aa 1-81. The humoral immune response was measured using PreS1-GST (aa 398) specific ELISA before and after each immunization. We examined the humoral immune response (PreS1-GST specific ELISA) prior to immunization and at week 2, 4 and 7 after beginning of immunizations (Fig. 22).

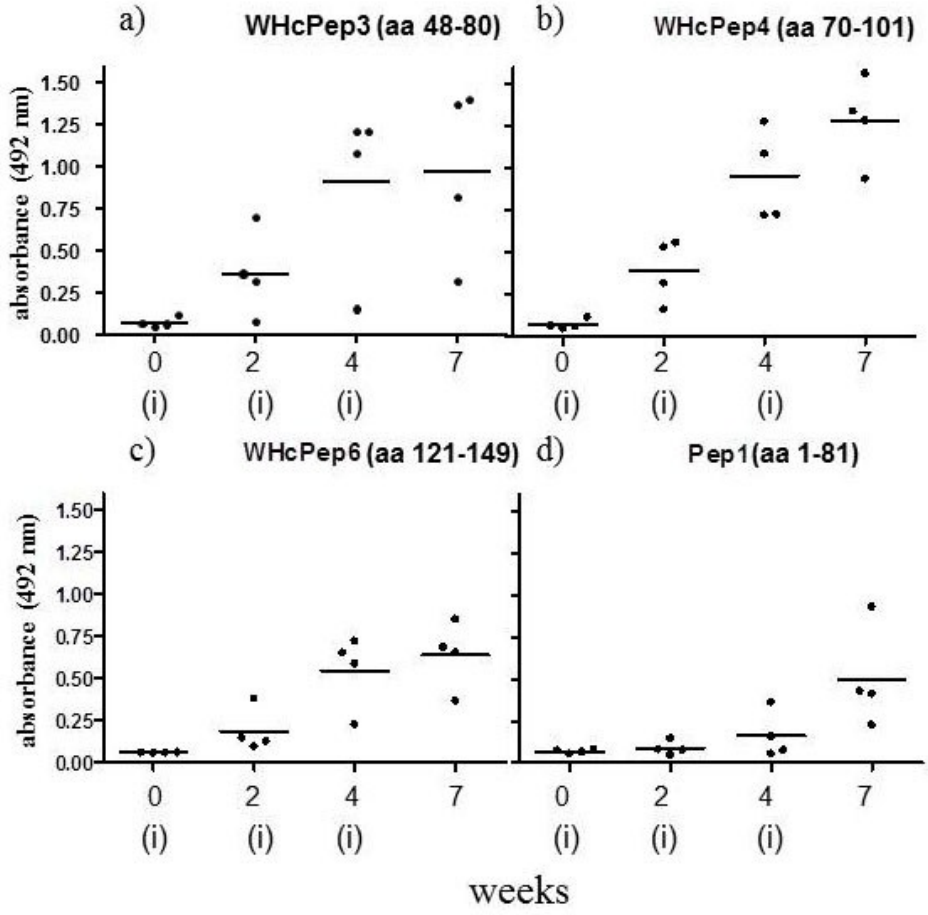

Figure 22: WHV PreS1-specific humoral immune response in WHcPep3, WHcPep4, WHcPep6 and Pep1-vaccinated mice.
BALB/c mice were immunized three times with construct WHcPep3, or WHcPep4, or WHcPep6 or peptide Pep1, respectively. After each immunization WHV PreS1-specific antibodies were determined. The mean value for each group is indicated by a bar.

Antibodies to PreS1 were undetectable prior to immunization (week 0) in all groups. After the first immunization all groups of mice immunized with VLPs showed PreS1 specific humoral immune response; whereas mice immunized with the PreS1 fragment aa 1-81 showed no immune response at this time point. This difference was statistically significant in case of WHcPep4 with P value of < 0.05. After second immunization two mice immunized with PreS1 fragment showed humoral immune response. In comparison all mice immunized with particles developed humoral immune response. The difference was also here statistically significant in case of WHcPep4. These results indicate that the produced particles induce earlier humoral immune response as compared to PreS1 fragment aa 1-81. After the last immunization all groups of mice developed humoral immune response to PreS1-GST. The neutralizing capacity of induced antibodies should still be tested.

In summary, we could show that WHV core VLPs produce a fast immune response already two weeks after the first immunization. These particles can now be used for immunization of woodchucks. Probably this vaccine can also be used for chronically infected woodchucks to check its ability for therapeutic vaccination.

4. Discussion

Liver transplantation is mostly the ultimate therapeutic option for patients with liver failure due to liver cirrhosis or hepatocellular carcinoma caused by HBV-, HCV-infection, autoimmune hepatitis or alcohol abuse. The lack of suitable organs led to an increased number of partial liver transplantations from living liver donors (LLD) (Broelsch et al., 2003). The HBV reinfection, however, is still a problem despite of a combined therapy with lamivudine and hepatitis B immunoglobulin (Lai et al., 1998; Tillmann et al., 1999). Adoptive transfer of HBV immunity with the liver of vaccinated donors could be a new approach to prevent reinfection in the recipients. In the last few decades several groups described detection of microchimerism after liver transplantation and a lymphocyte transfer within the transplanted liver (Schlitt et al., 1993). In countries with a high prevalence of HBV, the chance to transplant a liver from an immune donor is high and subsequent adoptive immune transfer is possible (Lo et al., 2003; Luo et al., 2007). In low prevalence areas like Europe, however, immune donors are rare. Therefore vaccination of LLD for adoptive immune transfer could be an alternative.

4.1. Short time immunization

The active HBV immunization of the donors for adoptive immune transfer with the liver graft has never been performed in humans. To date it is not clear to which degree HBV specific immunity, induced by immunization, can be transferred by liver transplantation. However, the adoptive transfer of humoral HBV specific immunity by liver transplantation has already been analyzed in the rat model (Dahmen et al., 2003a). After immunization, the rats displayed mean anti-HBs titers of approximately 100,000 IU/l. The corresponding non-immunized recipients showed anti-HBs titers of approximately 100 IU/l at week 1 after liver transplantation. These experiments indicate that only a small proportion of antibody-producing lymphocytes can be transferred to the recipient.

Because of the small amount of HBV specific B- and T-cells which can be transferred with the liver we expect that it is necessary to induce strong immune responses in the donors. However, the time in which to achieve this immune response is rather short (1-2 months). Therefore, we established a short time immunization protocol using Sci-B-Vac, a recently developed S, M and L protein containing HBV vaccine (Schumann et al., 2007). But the applicability of short time immunization protocols is not limited to liver donation settings.

Adolescents who are non- or low-responders after vaccination in a standard vaccination schedule (Wallace et al., 2004) and travelers who need HBV immunity within a short time would also profit from this vaccination scheme.

Sci-B-Vac has already been shown to induce stronger humoral immune responses compared to vaccines containing only the S protein. In the conventional HBV vaccination protocol (immunization at month 0, 1, and 6) with a vaccine dose of 10 µg Hourvitz et al. observed anti-HBs titers of 13,483 IU/l (GMT) after the third immunization with Sci-B-Vac at month 7 (Hourvitz et al., 1996). However, at month 12 the GMT decreased to 3,273 IU/l. In a short time immunization protocol (immunization at weeks 0, 2, 4, and 6) and a 2-fold higher vaccine dose (20 µg) we induced an anti-HBs titer of 843 IU/l (GMT) within 2 months.

Several placebo-controlled, double blind randomized trials showed safety of S containing vaccines by preventing hepatitis B in high risk groups (Szmuness et al., 1981; Coutinho et al., 1983). In general prevention from HBV infection is mostly achieved with anti-HBs titers higher than 10 IU/l. Our short time immunization protocol induced higher antibody titers which are relevant for the immediate protection against the virus.

Cellular immune response to the surface- and core-proteins in chronically infected patients is low or absent as compared to individuals resolving the infection or vaccinated individuals, respectively. Immunization of chronically infected patients with the S protein (HBVAXPRO®) cannot induce cellular immune responses (unpublished data, M. Fiedler and M. Lindemann). Cellular immune response to the core protein has already been shown to protect against infection in the woodchuck model (Roos et al., 1989; Menne et al., 1997; Lu et al., 1999). However, therapeutic vaccination with core protein in preclinical studies in chronically infected woodchucks was not efficient and vaccines containing the core protein are still not available for humans. Therefore, we used the highly immunogenic S, M and L protein containing vaccine as an alternative. Possibly the adoptive transfer of both humoral and cellular immune responses to the S, M and L proteins could be sufficient to control reinfection with the virus.

Similar to other available L, M and S protein-containing vaccines (Jones et al., 1998; McDermott et al., 1998; Jones et al., 1999) we could show cellular immune responses after vaccination with Sci-B-Vac. In addition to the conventionally used proliferation assay we measured the IFN-γ production by ELISpot. Thereby, the cellular immune response was

detected earlier (after the first vaccination) compared to the proliferation assay. In this assay proliferation of CD4 T-cells was detected after vaccination. Thus, IFN-γ ELISpot seems to be more sensitive for the early detection of the cellular immune response after vaccination with Sci-B-Vac.

To verify that the cellular immune response is also directed against PreS1 we decided to use a fragment of the L protein (108 aa) for stimulation. The Sci-B-Vac immunized volunteers showed cellular immune responses to that protein while the control group immunized with the S protein as expected did not. The L protein contains additional B- and T-cell epitopes and may induces neutralizing antibodies. The data on the stimulation with the S protein were comparable in the Sci-B-Vac and in the control group. Both vaccines contained this HBV protein.

4.2. Adoptive immune transfer

In the first part of this study a short time immunization protocol was established in order to induce high immune responses in LLD before transplantation and thereby to protect chronically HBV infected patients from reinfection of the liver graft by adoptive immune transfer. We have shown that vaccination with Sci-B-Vac induces strong immune responses in a short time immunization protocol (Schumann et al., 2007). Those immune responses, however, were lower in LLD compared to healthy volunteers. The reason for the reduced immune response could be psychological stress during the evaluation period of LLD. Several studies have already shown that mental pressure suppresses immune responses to HBV vaccines (Burns et al., 2002). In this part of the study, however, HBV specific adoptive transfer of humoral, and for the first time, of cellular immune responses with the human liver was demonstrated (Schumann et al., 2009). 14 of 46 immunized donors were accepted for donation. The transfer of the humoral and/or cellular immune responses was detected in 3 of 14 recipients. Two recipients were HBV naïve (recipients 1 and 2) and one was chronically HBV infected (recipient 3). In the corresponding donors anti-HBs titers > 9,000 IU/l were measured. Probably, due to the induction of intermediate anti-HBs titers 100 - 1,000 IU/l in 10/14 donors the transfer of immune responses to their corresponding recipients was not observed. The transfer of antibodies seems to depend on the level of the HBV specific humoral immunity in the donor prior to transplantation. Luo et al. have shown that particularly the humoral immune response (anti-HBs titers >1,000 IU/l) in the donor is essential for the adoptive immune transfer (Luo et al., 2007). In this study donors were not

vaccinated but had previous HBV infection. In our study, however, one donor with a high anti-HBs titer of 6,000 IU/l and additionally the highest cellular immune response did not transfer HBV specific immunity to the corresponding recipient. Possibly, immunity after infection is associated with a higher amount of HBV specific T-cells in the liver compared to that in livers of immunized donors. Therefore, immunized donors probably require higher member of B-cells to transfer their immunity than donors after resolved HBV infection. A calculation of minimal anti-HBs titer that is required for adoptive immune transfer from immunized donors is difficult.

In the chronically HBV infected recipient 3 an anti-HBs titer of 58,000 IU/l was measured after transplantation. Only about 4,700 IU/l of anti-HBs were calculated to be due to HBIg administration in recipient 3. In week 19 after transplantation HBV-DNA reappeared and anti-HBs was no longer measurable. This possibly indicates that adoptive transfer of HBV specific immunity was not sufficient in this patient to prevent graft reinfection. The state of health of this chronically HBV infected recipient, however, rapidly worsened due to the tumor at this time point. The patient died two months later. Probably, the development of a tumor impaired the function of the immune system. This aspect needs to be examined in a larger cohort of patients.

In order to clarify, if cells of the immunized donors or the recipients induced HBV specific immune responses we investigated the presence of microchimerism. In donor/recipient pair 1, cells of the male donor were not measurable by Y chromosome-specific PCR in the PBMCs of the female recipient one year after transplantation (data not shown). In other studies, it was shown that the frequency of transferred donor cells is mostly below 1% one month after transplantation and even lower thereafter (Jonsson et al., 1997; Ueda et al., 1997). Therefore we were not able to detect donors DNA by PCR. Microchimerism, however, still does not clarify the question, if the cells of the donor or of the recipient induce HBV specific B- and/or T-cell immune responses. Therefore, it would be necessary to evaluate a method to detect donor specific and simultaneously HBV specific cells in the recipient. Flow cytometry analysis allows the detection of donor/recipient specific HLA markers and simultaneously HBV specific cells. So far, however, we were not able to detect HBV specific B- and/or T-cells in the recipient after transplantation using flow cytometry analysis. On the one hand this could be due to the low frequency of HBV specific cells. Furthermore, it has been shown that detection of antigen specific T-cells by flow cytometry analysis is less sensitive than by ELISpot assay (Tassignon et al., 2005). Taken together, the evaluation of a protocol, which

can detect simultaneously donor specific and HBV specific cells, is not an easy task but an important goal to prove adoptive immune transfer by solid organ transplantation.

In conclusion, HBV specific adoptive immune transfer to transplanted patient due to chronic HBV infection in the setting of living liver transplantation is feasible. Our results clearly indicate that only high titers of anti-HBs in the donor result in immune transfer. It could be beneficial to extend the time for donor vaccination if possible. Hopefully in the future most possible donors are already immune due to vaccination programs for children in many countries. Further, a promising way to overcome the reinfection of the new graft in chronically HBV infected liver recipients could be the combination of adoptive immune transfer followed by booster immunizations. Either vaccines with highly immunogenic adjuvants like MPL/QS21 (Bienzle et al., 2003; Bauer et al., 2007) or third generation vaccines should be used (Lo et al., 2007). Further, the use of adoptive immune transfer could especially be useful for patients in countries where the medication with anti-HBs antibodies or/and improved vaccines is not available.

4.3. Characterization of WHV VLP

The worldwide prevalence of HBV clearly emphasizes need of prophylactic vaccination and improved therapy in patients with chronic HBV infection. Vaccines against HBV are available since 1980s. Protective immune response, however, can be reached in about 90 – 95% of all vaccine recipients. Therefore one aim of this thesis was to characterize the vaccine improvement by HBV PreS1 protein. One important function of PreS1 protein is binding to the NTCP receptor of cells to enable the entry of the virus into host which is mainly the hepatocyte. The n-terminal part of PreS1 was considered as the receptor binding site (Meier et al., 2013) and recently the receptor was identified (Yan et al., 2012; Zhong et al., 2013).
Hepadnaviral core protein offers the ability to act either as T-cell and B-cell antigen. Thereby a vigorous T-cell immune responses and various repertoire of antibodies can be induced.
Therefore PreS1 in combination with hepadnaviral core protein virus-like particles (VLP) represent an interesting approach for prophylactic hepatitis B vaccine. Thereby, T-cell dependent immune responses and a broader range of neutralizing antibodies could be induced resembling the repertoire of antibodies acquired during natural infection (Neurath et al., 1987).
Purification of HBV PreS1-core particles in E. coli was successful performed by two groups (Kazaks et al., 2004; Malik et al., 2012). In addition the ability to induce antibodies was approved in mice. Unfortunately, mouse is not the natural host of HBV and can therefore not be infected with the virus. Thus, the protecting proprieties of HBV PreS1-core particles as therapeutic and/or prophylactic vaccine could not be tested in vivo. In vitro, however, *Tupaia belangeri* hepatocytes which can be infected with HBV were used for testing of neutralizing capacity of these antibodies (Glebe et al., 2003). *Tupaia belangeri*, however is not the natural host of hepadnaviruses and could not be used for protection studies in vivo.

As a preclinical in vivo model we used therefore the woodchuck (*Marmota monax*) which can be infected with the woodchuck hepatitis virus (WHV). In contrast to HBV, the PreS1 binding side of WHV and the receptor in the host was not identified so far. Therefore overlapping peptides (Pep1-Pep6) bearing the whole PreS1 sequence were used to create six VLPs. The PreS1 region, which induces neutralizing antibodies in the woodchuck model, should be identified. Meanwhile protection against WHV using the WHV PreS1 fragment aa 1-81 as antigen was successfully performed (Schumann and Brovko et al., unpublished data). This finding is in accordance with HBV PreS1 in vitro studies performed in the past (Ryu et al., 1997; Hong et al., 2004; Glebe et al., 2005; Glebe, 2006). Further, WHV core antigen alone

has been shown to protect woodchucks from WHV infection (Menne et al., 1997). Acute WHV infection in woodchucks can furthermore similar to HBV change to a chronic disease. This model is therefore suitable to examine not only prophylactic but as well therapeutic ability of PreS1-core particles.

In this study WHV core particles containing WHV PreS1 inserts were created similar to HBV particles constructed by Borisova (Borisova et al., 1990). The major immunodominant region (MIR) of WHV core protein was identified using a prediction system based on the known HBV core protein structure (Wynne et al., 1999; Tan et al., 2007). Based on this prediction, PreS1 peptides were inserted between aa 78 and 79 of the WHV core protein. Meanwhile, Billaud et al. have shown that WHcAg appears to tolerate insertions of foreign epitopes at a greater length and different positions than HBV core protein (Billaud et al., 2005a; Billaud et al., 2005b). They predicted aa 78/79 of the WHV core as a tolerant insertion site for insertion of foreign epitopes. These findings correlate with our data, since analysis of the bacterial lysates demonstrated that all six chimeric WHV core-PreS1 proteins were assembled into VLP (Fig 20). Unfortunately we were not able to purify all six PreS1-core particles. However, the PreS1-core particles WHcPep3, WHcPep4 and WHcPep6 were purified. Immunization of mice with chimeric particles purified in this study (WHcPep3, WHcPep4, WHcPep6) induced high antibody tiers which were better than after immunization with the n-terminal PreS1-fragment aa 1-81. Meantime Brovko et al. purified core particles using a Split-core system based on HBV core protein (Walker et al., 2011) and purified particles containing the WHV PreS1 region aa 22−38 (Brovko, 2012). Further a WHV PreS1 B-cell epitope (aa 22-25) was identified. Similar to our results, however, split-core-particles based on WHV core protein bearing the PreS1 region 22-38 could not be purified.

Finally, particles created in this study can be used in the woodchuck model to confirm the protection ability and safety of this vaccine for application of HBV VLP in humans.

4.4. Future Prospects

VLPs developed in this thesis induce core specific T- and B-cell immune response against Hepadnaviral infection and simultaneously improve immunogenicity of PreS1 by elevating the titer of PreS1-specific antibodies. Probably, a PreS1-insert smaller in length can improve the solubility and particularly purification ability of the chimeric WHV PreS1-core VLPs. Meantime a WHV PreS1 B-cell epitope aa 22-25 was identified (Brovko, 2012). Chimeric WHV core particles containing this epitope should be developed to examine their sufficiency in therapeutic vaccination of individuals suffering from chronic hepadnaviral infection.

Chimeric particles created in this study, however, can be used to confirm its safety and protection ability in vivo using woodchucks as animal model.

5. Literature

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6. Summary

Liver transplantation is often the ultimate option of therapy for chronically hepatitis B virus (HBV) infected patients. Prevention of reinfection by HBIg is long-lasting and cost-intensive. Adoptive transfer of HBV immunity after transplantation of the liver from an immune living liver donor (LLD) could be a new approach to prevent reinfection in the recipients.

A short time immunization protocol (four injections in 2 weeks intervals) was established to achieve HBV immunity in LLD within 2 months. Using this protocol, the humoral and cellular immune response of Sci-B-Vac, a recombinant vaccine that contains HBV L, M and S surface proteins was compared with a standard HBV vaccine (HBVAXPRO®) that contains only the S surface protein. The humoral immune response could already be detected after the first immunization in nine out of fifteen Sci-B-Vac vaccinated individuals while it was only observed in one out of fifteen volunteers of the group immunized with HBVAXPRO®. Anti-HBs titers were significantly higher in Sci-B-Vac volunteers ($P < 0.01$) following all four vaccinations. HBV-specific T-cell immune response was significantly higher in Sci-B-Vac volunteers ($P < 0.001$) after the third vaccination. Proliferative response was also significantly ($P < 0.01$) higher in the Sci-B-Vac group after second to fourth vaccination.

With this good experience, Sci-B-Vac was subsequently used for vaccination of forty-six potential LLD using the short time immunization protocol as described above. Humoral and cellular immune responses were examined in donors after immunization and in recipients before and after transplantation. Anti-HBs-titers of up to 50,000 IU/l were detected in LLD. Fourteen patients received livers from these immunized donors. We detected humoral immunity in one HBV-naïve recipient and in one chronically HBV-infected recipient. A transfer of cellular immunity ($SI > 3$) was seen in three recipients. These three patients received livers from donors with high anti-HBs-titers of more than 9,000 IU/l. Cellular immunity was also detected in the corresponding donors ($SI > 3$ and spots > 22). Our study demonstrates that HBV-specific humoral and cellular immunity can be transferred by liver transplantation after vaccination of the donors. The transfer of B- and T-cell immunity in transplanted patients correlates with the magnitude of immune responses in the donor.

Further, using the woodchuck model we successfully expressed six chimeric PreS1-core particles (WHcPep1, WHcPep2, WHcPep3, WHcPep4, WHcPep5, WHcPep6) in *E. coli*. We successfully purified three out of six constructs (WHcPep3, WHcPep4, WHcPep6). Immunization of mice with chimeric particles purified in this study induced a better immune response compared to immunization with an n-terminal PreS1-fragment aa 1-81. Already, after the first immunization all groups of mice immunized with virus like particles (VLPs)

showed PreS1 specific humoral immune response; whereas mice immunized with the PreS1 fragment aa 1-81 showed no immune response at this time point. Particles created in this study can be used in the future in woodchucks to confirm the protection ability and safety of this vaccine for application of HBV VLPs in humans.

7. Zusammenfassung

Die Lebertransplantation ist die letzte Therapiemöglichkeit für chronisch Hepatitis-B-Virus (HBV) infizierte Patienten. Die Prävention einer HBV-Reinfektion durch Immunglobuline ist langwierig und teuer. Eine mögliche Alternative die Reinfektion zu verhindern könnte der adoptive Transfer von HBV-Immunität durch Lebertransplantation nach Impfung eines Leber-Lebendspenders sein.

Im ersten Teil dieser Arbeit wurde ein Kurzzeit-Immunisierungsprotokoll (vier Impfungen in 2-wöchigen Abständen) etabliert, um eine HBV-spezifische Immunantwort innerhalb von 2 Monaten zu induzieren. Bei diesem Immunisierungs-Schema wurde die humorale und zelluläre Immunantwort nach Impfung mit Sci-B-Vac, einem rekombinanten Impfstoff, der aus den Oberflächenproteinen L, M und S besteht, verglichen mit dem Standardimpfstoff, der nur das S Protein besitzt (HBVAXPRO®). Bei neun von fünfzehn Impflingen der Sci-B-Vac-Gruppe konnte bereits zwei Wochen nach der ersten Impfung eine humorale Immunantwort detektiert werden. Bei den HBVAXPRO®- Impflingen konnte zu diesem Zeitpunkt bei nur einem von 15 Probanden eine humorale Immunantwort nachgewiesen werden. Die anti-HBs Titer waren in der Sci-B-Vac-Gruppe nach der ersten bis vierten Impfung signifikant höher ($P < 0.01$). Die HBV-spezifische T-Zellproliferation war in der Sci-B-Vac-Gruppe nach der zweiten, dritten und vierten Impfung signifikant höher ($P < 0.01$).

Aufgrund der guten Ergebnisse mit dem Impfstoff Sci-B-Vac wurden 46 potentielle Leber-Lebendspender nach dem oben beschriebenen Kurzzeit-Immunisierungsprotokoll geimpft. Die humorale und zelluläre Immunantwort wurde bei den Spendern nach Impfung und bei den Empfängern vor und nach Transplantation gemessen. Anti-HBs Titer von bis zu 50000 IU/l wurden bei den Spendern nach Impfung detektiert. Vierzehn Empfänger haben ein Teil der Leber der geimpften Leber-Lebendspender erhalten. Eine humorale Immunantwort wurde nach Transplantation bei einem vorher HBV-naiven Empfänger und bei einem chronisch HBV-infizierten Empfänger nachgewiesen. Ein Transfer der zellulären Immunität (SI > 3) wurde bei drei Empfängern detektiert. Diese drei Patienten hatten Lebern von Spendern erhalten, deren anti-HBs Titer höher als 9000 IU/l waren. Die zelluläre Immunantwort war bei den korrespondierenden drei Spendern ebenfalls kräftig [(SI > 3 und HBV-spezifische Interferon-γ Spots > 22 (ELISpot)]. Diese Studie zeigt, dass die humorale und zelluläre Immunantwort nach Impfung der Spender mit der Leber übertragen werden kann. Der Transfer von B- und T-Zell-Immunität korreliert dabei mit der Stärke der Immunantwort des Spenders.

Weiterhin wurden im Murmeltier-Model sechs Virus-ähnliche-Partikel (VLP) konstruiert. Diese Partikel beinhalten neben dem Core-Protein auch Peptide aus der PreS1-Sequenz des

Woodchuck-Hepatitis-Virus. Drei von sechs VLP (WHcPep3, WHcPep4, WHcPep6) konnten aus *E. coli* Lysat gereinigt werden. Die Immunisierung von Mäusen zeigte, dass VLP eine bessere Immunantwort induzieren als eine Peptid-Immunisierung mit dem n-terminalen PreS1-Fragment (aa 1-81). Bereits nach der ersten Impfung haben alle Mäuse, die mit VLP geimpft wurden, eine humorale Immunantwort gegen das PreS1-Protein entwickelt. Mäuse, die mit dem PreS1-Peptid (aa 1-81) geimpft wurden, zeigten zu diesem Zeitpunkt keine PreS1-spezifische Immunantwort. Die in dieser Studie hergestellten WHV Partikel können auf ihre Sicherheit und die Induktion protektiver Immunität untersucht werden, um in Zukunft HBV Partikel in klinischen Studien anzuwenden.

8. Appendix

8.1. Plasmid maps

Maps of vectors used during the experiments:

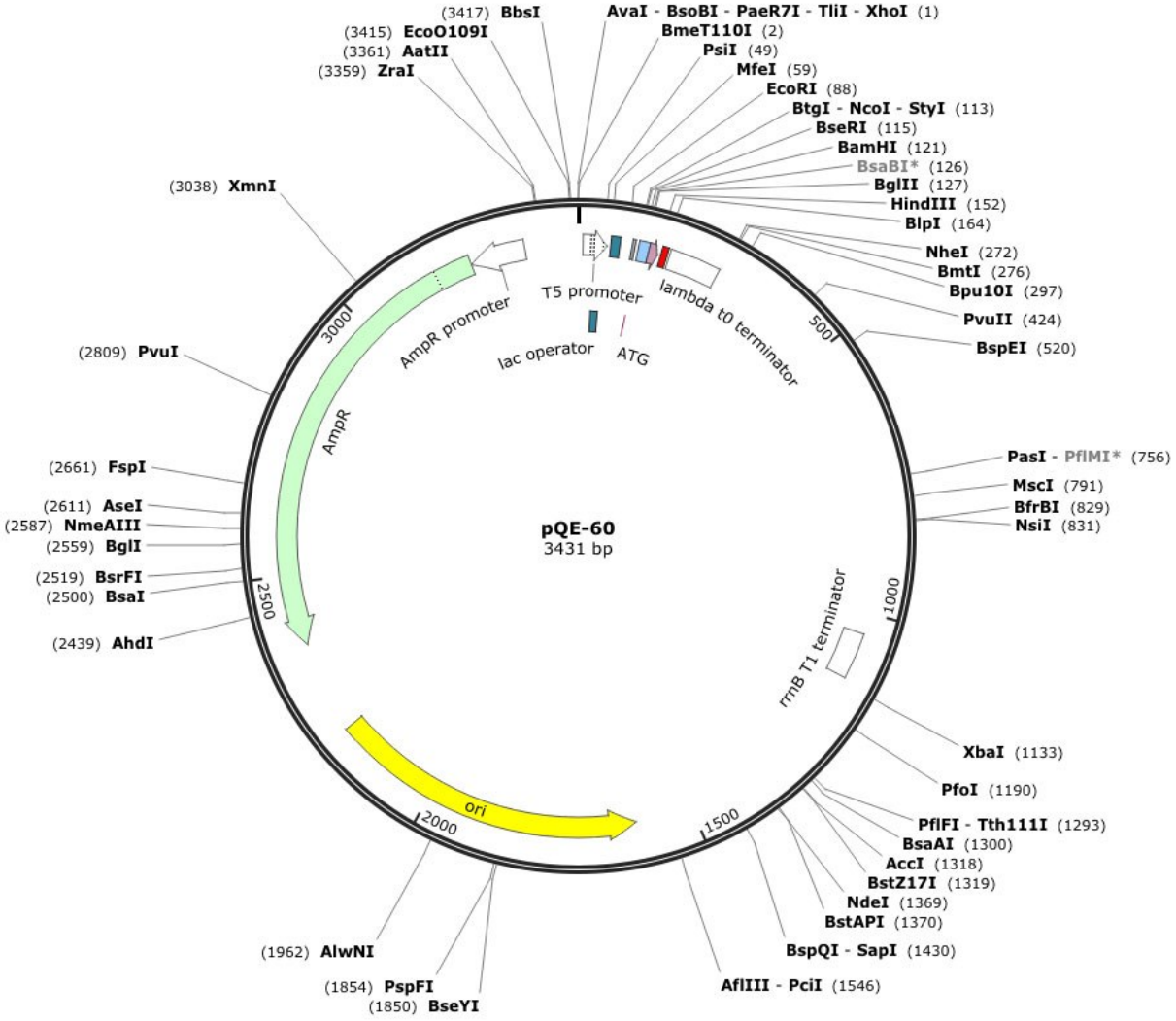

Figure 23: Schematic map of PQE60.
PQE60 plasmid was used for bacterial expression of the 149aa truncated wildtype WHcAg and for expression of WHcPep1-WHcPep6. The plasmid consists of: a c-terminally 6xHis tagged insert; restriction enzymes; T5 promoter and lac operator element; lambda transcription termination region; rrnB transcription termination region; ColE1-type origin of replication; ampicillin resistance gene (beta-lactamase cds).

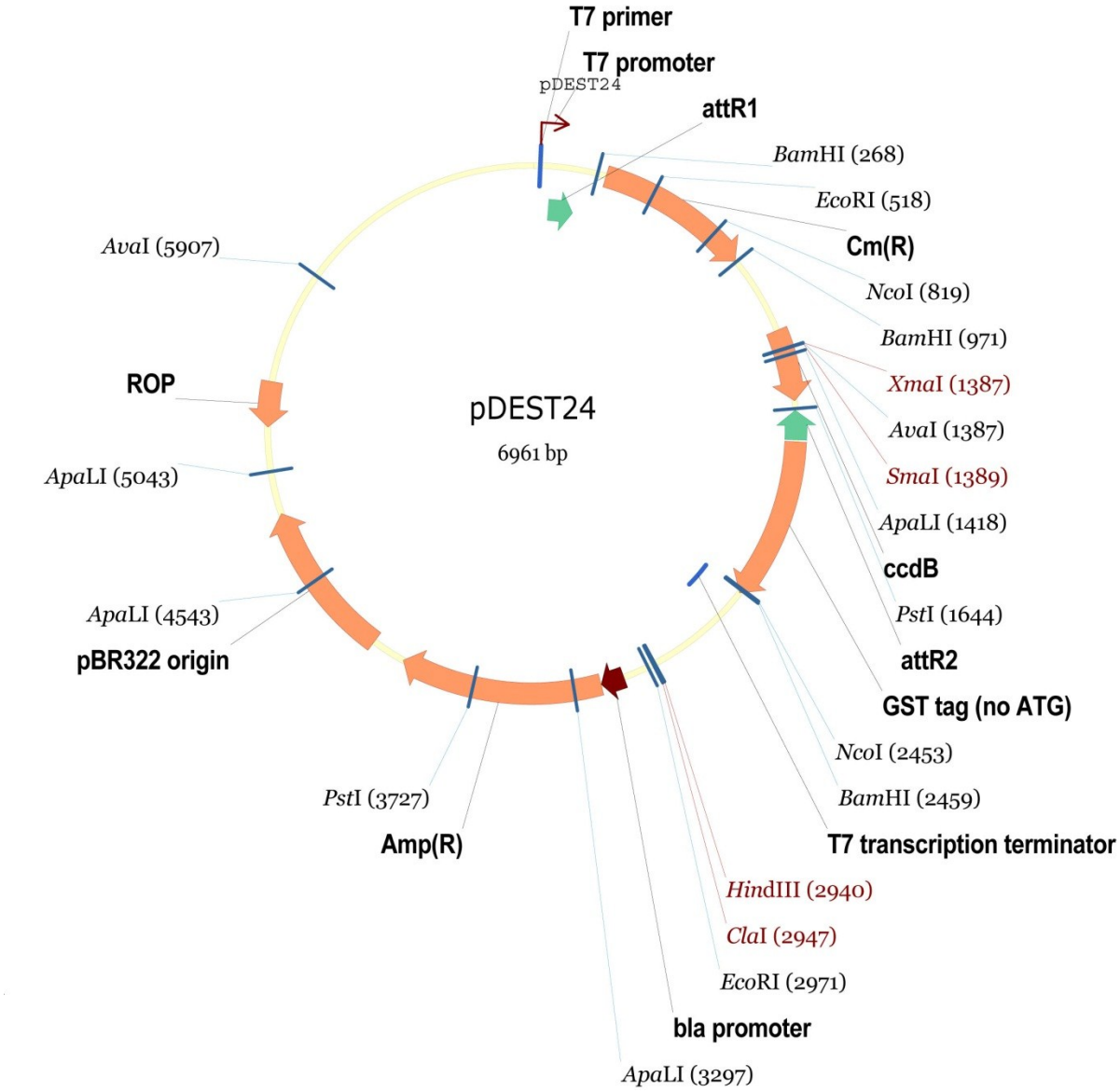

Figure 24: Schematic map of pDEST24.
pDEST24 plasmid was used for bacterial expression of the PreS1-GST protein. The plasmid belongs to Invitrogen Gateway technology plasmids. The plasmid consists of: a c-terminal GST insert; restriction enzymes; T7 promoter; T7 transcription termination region; pBR322 origin of replication; ampicillin resistance gene.

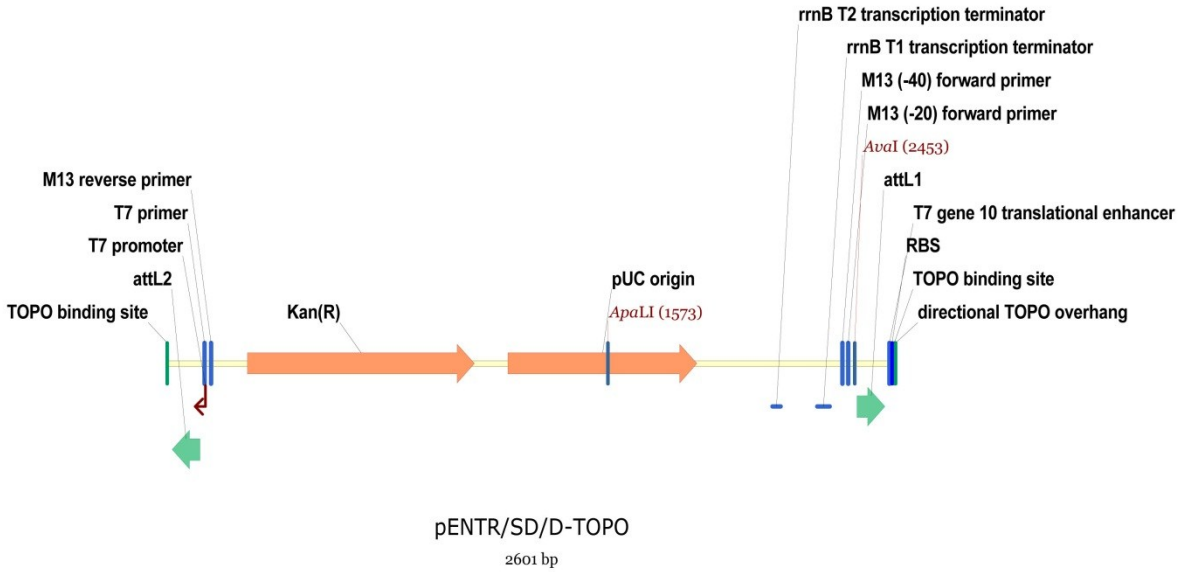

Figure 25: Schematic map of pENTR/SD/D-TOPO.
pENTR/SD/D-TOPO plasmid was used for cloning of preS1 gene into pDEST24 plasmid. The plasmid consists of: TOPO binding site; T7 promoter; rrnB T1 and rrnB T2 transcription termination region; pUC origin of replication; kanamycin resistance gene.

8.2. Protein- and DNA- sequences

Protein- and DNA-sequences used and generated during the experiments:

```
+1   Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ser Ser Tyr Gln Leu Leu Asn Phe Leu Pro Leu Asp Phe Phe Pro Asp Leu Asn Ala Leu Val Asp Thr Ala
1    ATGGACATAG ATCCCTATAA AGAATTTGGT TCATCTTATC AGTTGTTGAA TTTTCTTCCT TTGGACTTCT TTCCTGACCT TAATGCTTTG GTGGACACTG
     TACCTGTATC TAGGGATATT TCTTAAACCA AGTAGAATAG TCAACAACTT AAAAGAAGGA AACCTGAAGA AAGGACTGGA ATTACGAAAC CACCTGTGAC

                                                                                                    HindIII
+1   Ala Thr Ala Leu Tyr Glu Glu Glu Leu Thr Gly Arg Glu His Cys Ser Pro His His Thr Ala Ile Arg Gln Ala Leu Val Cys Trp Asp Glu Leu Thr Lys
101  CTACTGCCTT GTATGAAGAA GAGCTAACAG GTAGGGAACA TTGCTCTCCG CACCATACAG CTATTAGACA AGCTTTAGTA TGCTGGGATG AATTAACTAA
     GATGACGGAA CATACTTCTT CTCGATTGTC CATCCCTTGT AACGAGAGGC GTGGTATGTC GATAATCTGT TCGAAATCAT ACGACCCTAC TTAATTGATT
+1   Lys Leu Ile Ala Trp Met Ser Ser Asn Ile Thr Ser Glu Gln Val Arg Thr Ile Ile Val Asn His Val Asn Asp Thr Trp Gly Leu Lys Val Arg Gln Ser
201  ATTGATAGCT TGGATGAGCT CTAACATAAC TTCTGAACAA GTAAGAACAA TCATAGTAAA TCATGTCAAT GATACCTGGG GACTTAAGGT GAGACAAAGT
     TAACTATCGA ACCTACTCGA GATTGTATTG AAGACTTGTT CATTCTTGTT AGTATCATTT AGTACAGTTA CTATGGACCC CTGAATTCCA CTCTGTTTCA
+1   Leu Trp Phe His Leu Ser Cys Leu Thr Phe Gly Gln His Thr Val Gln Glu Phe Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Ala Pro Tyr Arg Pro
301  TTATGGTTTC ATTTGTCATG TCTCACTTTC GGACAACATA CAGTTCAAGA ATTTTTAGTA AGTTTTGGAG TATGGATCAG AACTCCAGCT CCATATAGAC
     AATACCAAAG TAAACAGTAC AGAGTGAAAG CCTGTTGTAT GTCAAGTTCT TAAAAATCAT TCAAAACCTC ATACCTAGTC TTGAGGTCGA GGTATATCTG
+1   Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu His Thr Val Ile
401  CTCCTAATGC ACCCATTCTC TCGACTCTTC CGGAACATAC AGTCATT
     GAGGATTACG TGGGTAAGAG AGCTGAGAAG GCCTTGTATG TCAGTAA
```

Figure 26: WHcAg DNA and protein-sequence.
For expression PQE60 plasmid was used.

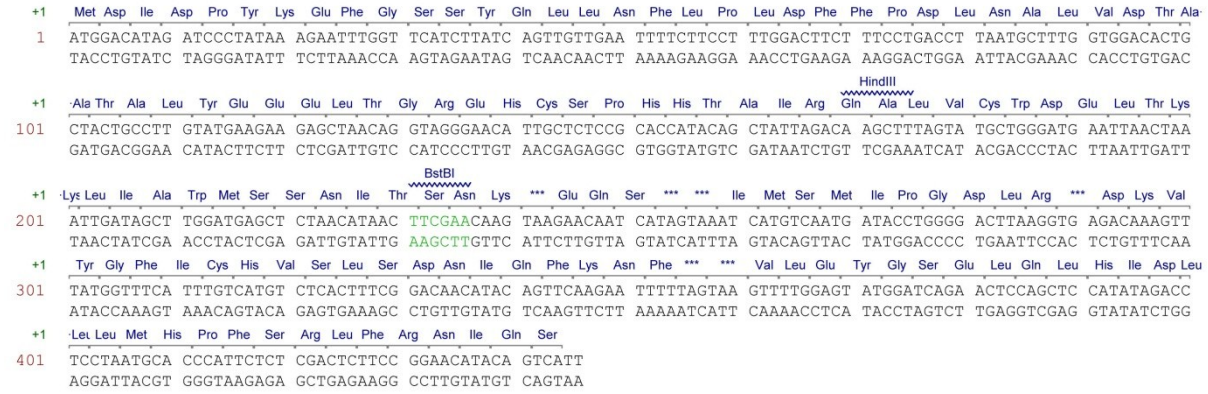

Figure 27: WHcAg DNA and protein-sequence including Bsp119 restriction site.
The PQE60 plasmid including this sequence was used for cloning of PreS1 peptides Pep1-Pep6 into the MIR of WHcAg. Restriction site Bsp119 (BstBI) is highlighted in green.

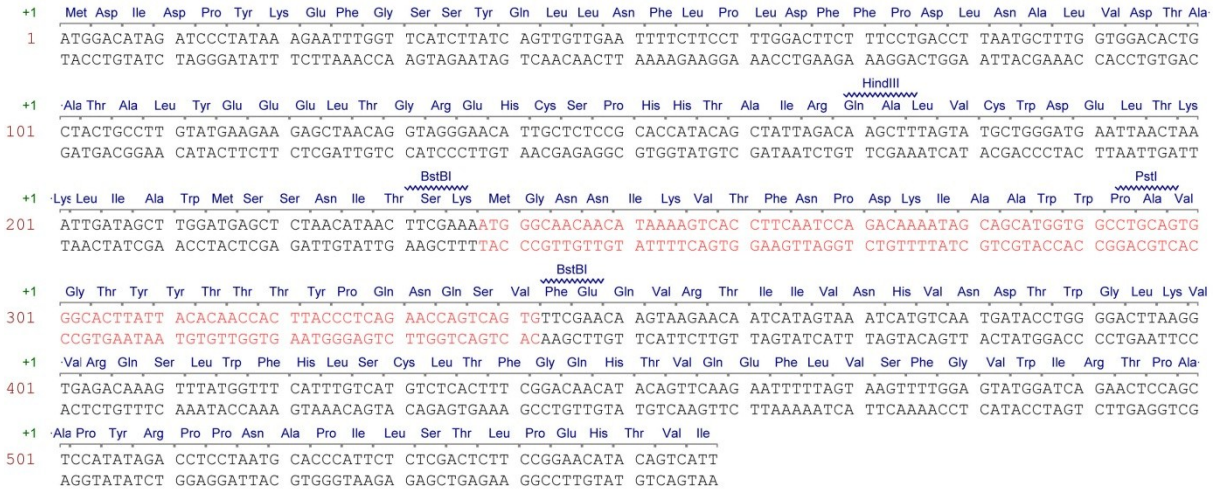

Figure 28: WHcPep1 DNA and protein-sequence.
The PQE60 plasmid including this sequence was used for protein expression. The PreS1 peptide Pep1 is highlighted in red.

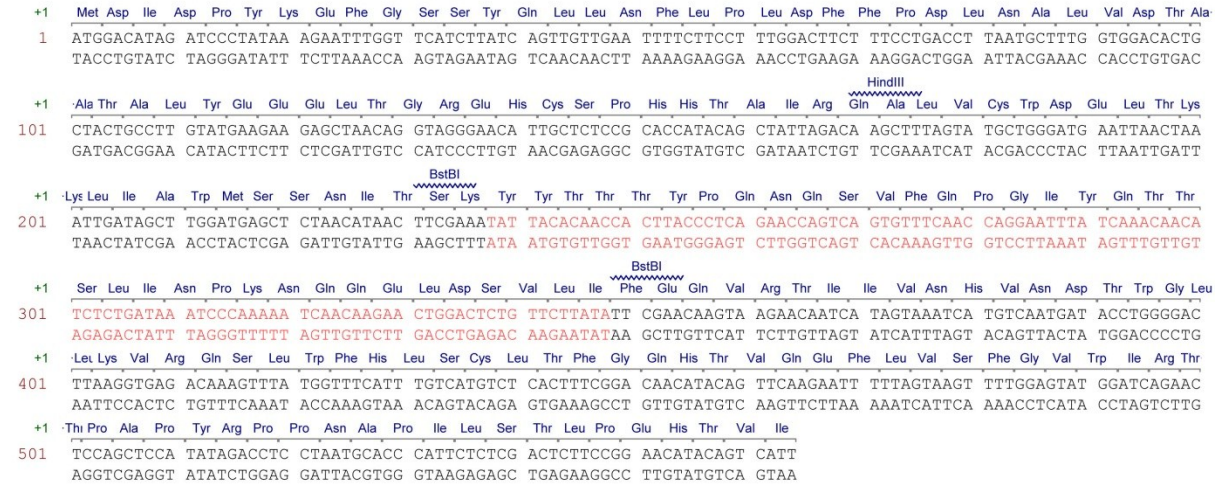

Figure 29: WHcPep2 DNA and protein-sequence.
The PQE60 plasmid including this sequence was used for protein expression. The PreS1 peptide Pep2 is highlighted in red.

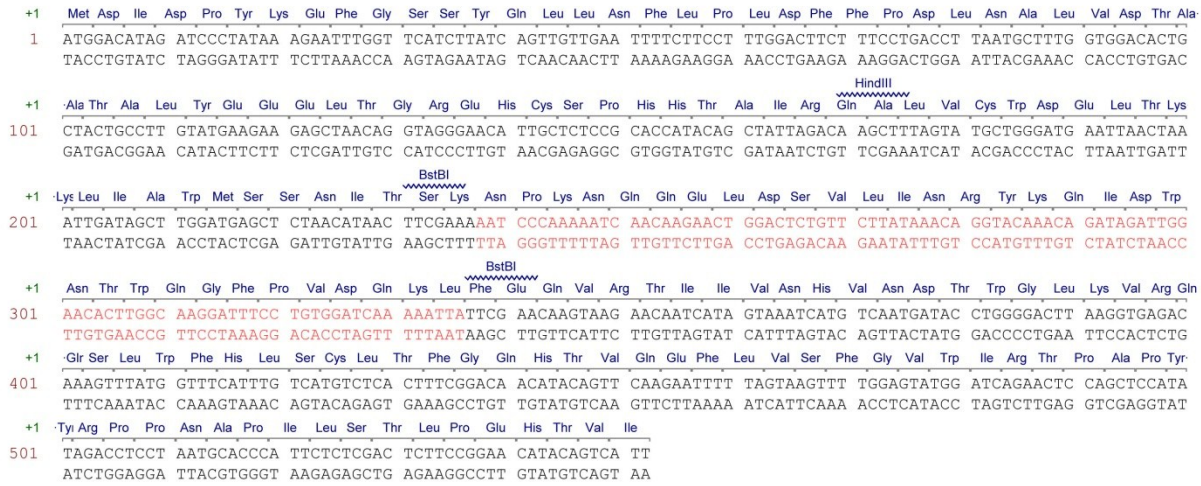

Figure 30: WHcPep3 DNA and protein-sequence.
The PQE60 plasmid including this sequence was used for protein expression. The PreS1 peptide Pep3 is highlighted in red.

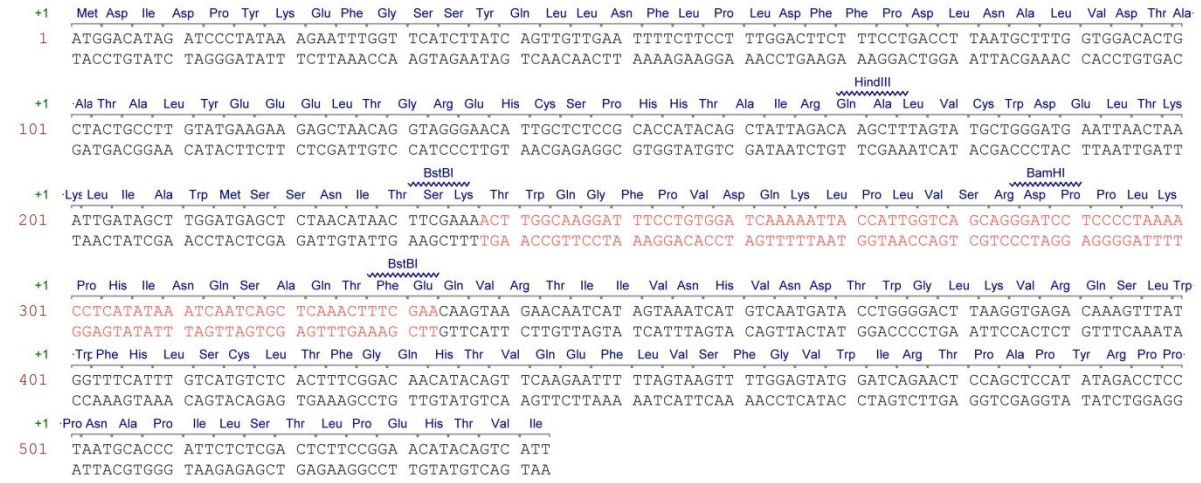

Figure 31: WHcPep4 DNA and protein-sequence.
The PQE60 plasmid including this sequence was used for protein expression. The PreS1 peptide Pep4 is highlighted in red.

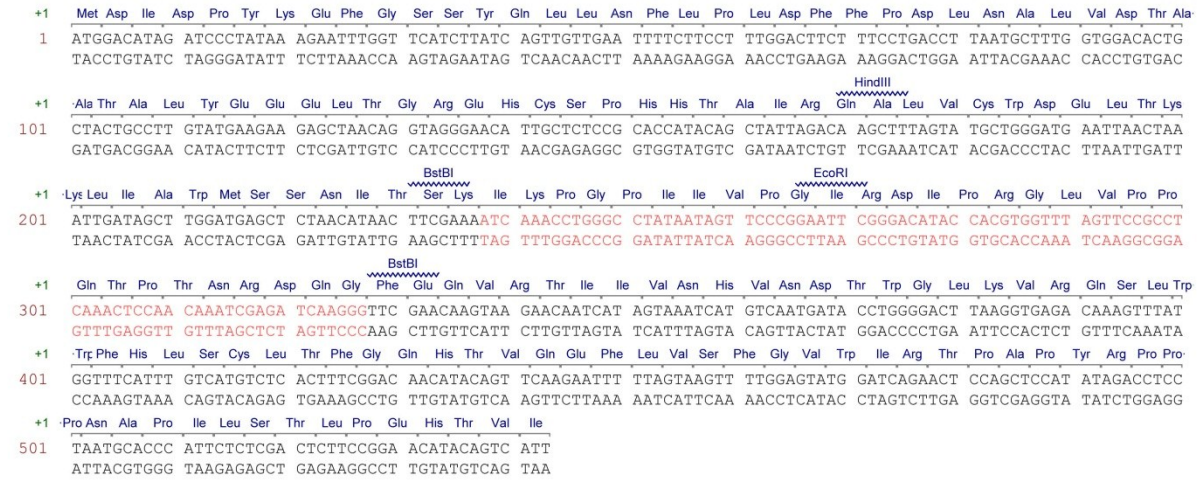

Figure 32: WHcPep5 DNA and protein-sequence.
The PQE60 plasmid including this sequence was used for protein expression. The PreS1 peptide Pep5 is highlighted in red.

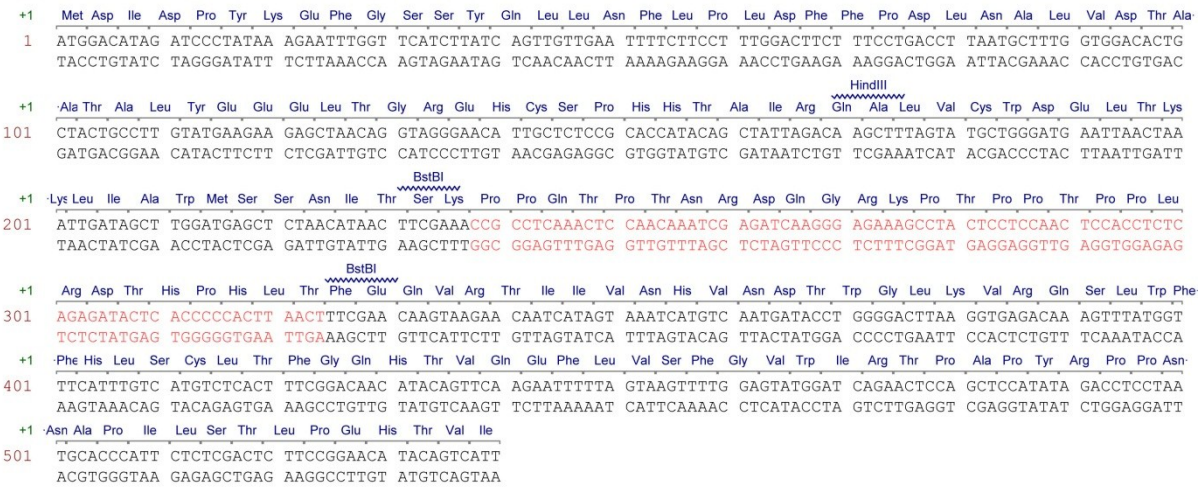

Figure 33: WHcPep6 DNA and protein-sequence.
The PQE60 plasmid including this sequence was used for protein expression. The PreS1 peptide Pep6 is highlighted in red.

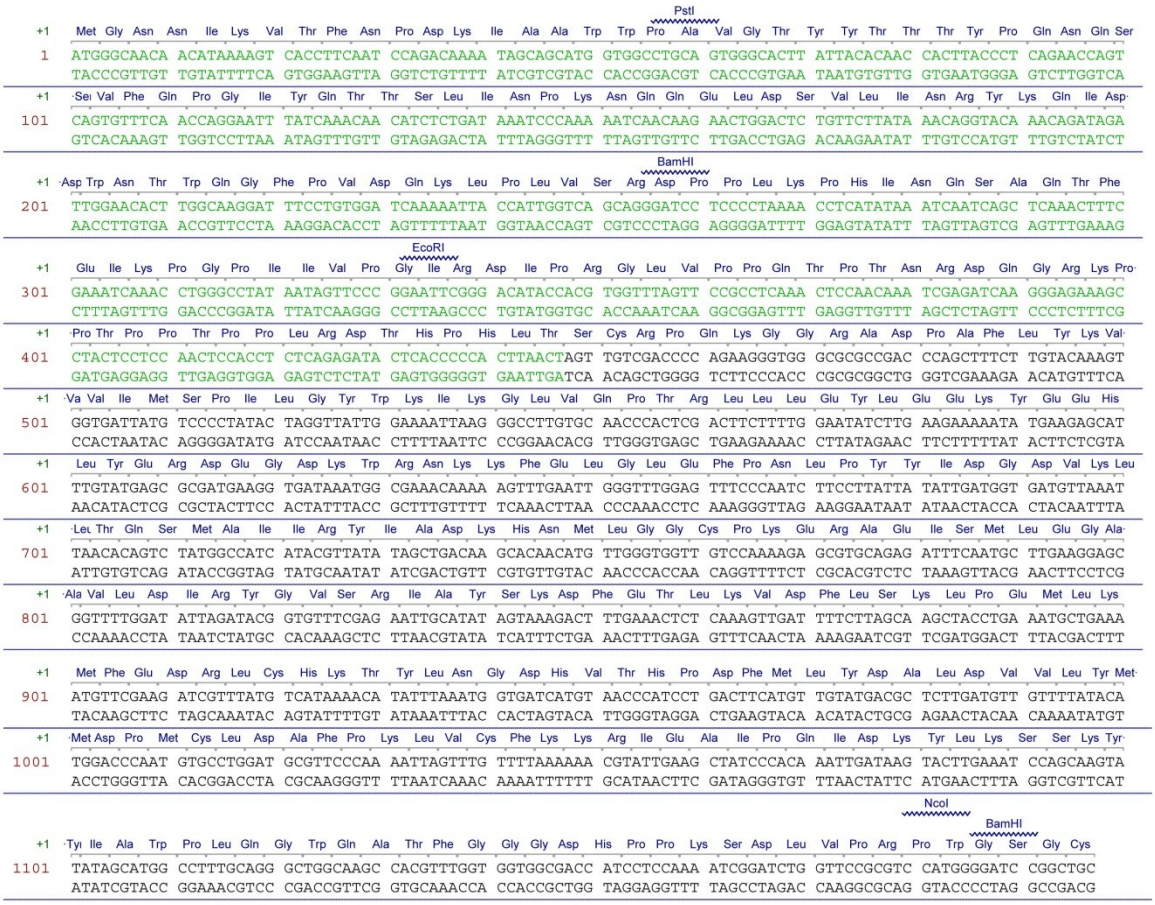

Figure 34: PreS1-GST DNA and protein-sequence.
The Gateway pDEST24 plasmid including this sequence was used for protein expression. The PreS1 fragment is highlighted in green.

9. Acknowledgement

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I express my last words to my dear husband. Thank you for your unresting support and your unlimited love.

10. Curriculum Vitae

11. Publications

1. Fiedler M, Kosinska A, **Schumann A**, Brovko O, Walker A, Lu M, Johrden L, Mayer A, Wildner O, Roggendorf M. Prime/boost immunization with DNA and adenoviral vectors protects from hepatitis D virus (HDV) infection after simultaneous infection with HDV and woodchuck hepatitis virus. J Virol. 2013 Jul; 87 (13): 7708-16.
2. **Schumann, A**., Lindemann M, Valentin-Gamazo C, Lu M, Elmaagacli A, Dahmen U, Knop D, Broelsch CE, Grosse-Wilde H, Roggendorf M, Fiedler M. Adoptive immune transfer of hepatitis B virus specific immunity from immunized living liver donors to liver recipients. Transplantation, 2009. 87 (1): 103-11.
3. Hitziger T, Schmidt M, Schottstedt V, Hennig H, **Schumann A**, Ross S, Lu M, Seifried E, Roggendorf M. Cellular immune response to hepatitis C virus (HCV) in nonviremic blood donors with indeterminate anti-HCV reactivity. Transfusion, 2009. 49 (7): 1306-13.
4. **Schumann A**., Fiedler M, Dahmen U, Grosse-Wilde H, Roggendorf M, Lindemann M. Cellular and humoral immune response to a third generation hepatitis B vaccine. J Viral Hepat, 2007. 14 (8): 592-8.
5. Sorg U.R., Kleff V, Fanaei S, **Schumann A**, Moellmann M, Opalka B, Thomale J, Moritz T. O6-methylguanine-DNA-methyltransferase (MGMT) gene therapy targeting haematopoietic stem cells: studies addressing safety issues. DNA Repair (Amst), 2007. 6 (8): 1197-209.
6. Jagiello P, Hammans C, Wieczorek S, Arning L, **Stefanski A**, Strehl H, Epplen JT, Gencik M. A novel splice site mutation in the TRIM37 gene causes mulibrey nanism in a Turkish family with phenotypic heterogeneity. Hum Mutat, 2003. 21 (6): 630-5.

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, f der Promotionsordnung der Math. – Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Hepatitis B virus specific adoptive immune transfer in living liver donation and characterization of a prophylactic/therapeutic vaccine against Hepadnaviral infection“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Alexandra Schumann befürworte.

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